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Alasdair M. Gilfillan
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Mast Cell Biology

Contemporary and Emerging Topics

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Volume 716
MAST CELL BIOLOGY: CONTEMPORARY AND EMERGING TOPICS
Alasdair M. Gilfillan and Dean D. Metcalfe

Mast Cell Biology

Contemporary and Emerging Topics

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DEDICATION

The editors would like to dedicate this work to the many present and former members of the Laboratory of Allergic Diseases who have contributed to the success of the clinical and basic research programs in the LAD since the inception of the Laboratory in 1995. We feel privileged to have worked with so many talented and enthusiastic fellows, nurses, administrators and senior scientists. A special thanks to Dr. Anthony Fauci, the Director of the National Institute of Allergy and Infectious Diseases who, along with Dr. Tom Kindt, then the Director of the NIAID Division of Intramural Research, supported the creation of the LAD; and to Dr. Katherine Zoon for her encouragement and support in the contemporary expansion of the clinical research program of the LAD. We are especially indebted to the patients who have participated in clinical research protocols directed at understanding and treating allergic diseases and systemic mast cell disorders.

FOREWORD

The editors of this book, Drs. Gilfillan and Metcalfe, have enlisted an outstanding group of investigators to discuss the emerging concepts in mast cell biology with respect to development of these cells, their homeostasis, their activation, as well as their roles in maintaining health on the one hand and on the other, their participation in disease.

As noted by several of the contributors, there have been extraordinary advances in our understanding of these phenomena over the past 40 years since the tools became available to analyze these aspects at a molecular level. Initially, this ability was made possible by the use of so-called model systems by which phenomena such as the allergic response involving the IgE-mediated activation of mast cells, and the related basophils, could be investigated. The discovery of a rat mucosal mast cell tumor analog –the "rat basophilic leukemia" (RBL) cells—and the development of lines of IgE myeloma protein producing cell lines, permitted a rigorous analysis of the cellular receptor that triggered the explosive release of a variety of potent mediators. This was soon followed by the initial identification of the proximal post-receptor molecules that were activated when the receptor-bound IgE was aggregated by antigen or bivalent anti-IgE. As knowledge about similar systems expanded, extraordinarily powerful cell biological methods were developed. That knowledge and those methods have been productively applied to analyze those model systems and, increasingly, their normal counterparts in rodent cells and, even more recently, in human cells. At the same time, less reductive more physiological studies have vastly increased our insight into the role mast cells play in a variety of aspects of the immune response.

These new insights not only expand our knowledge of cell biology in general but hopefully will be therapeutically applicable. Indeed, almost without exception, the authors of each of the articles in this compendium end their presentations by noting that their studies have helped to define new targets to which drugs could be directed in order to alleviate some of the pathological phenomena that are mediated by mast cells. However, as noted for example in the contribution by Tsai et al, the fact that mast cells have both positive and negative immunomodulatory functions present the challenge of whether such functions can be manipulated for therapeutic ends by suppressing those

actions that result in disease without interfering with those functions that promote health by enhancing beneficial immune responses.

Two recent events underscore this dilemma. On the down side is the case of Alzheimer disease. It has long been posited that the disease is caused by the accumulation of so-called amyloid beta plaques in the brain. Because gamma secretase is believed to play an important role in formation of the plaque, a drug designed to inhibit the activity of that enzyme was thought to be promising. Indeed, a trial of such a drug, Eli Lilly's semagacestat, had advanced to a Phase III clinical trial involving some 2600 patients. But in August of this year the multi-million dollar trial had to be abruptly halted when the developing data showed that the drug not only failed to slow progression of the disease, but was associated with a worsening of cognition and the ability to perform the tasks of daily living.

On the other hand, a more hopeful conclusion is prompted by a report published that same month by the laboratory of Tak Mak.[1] That group has been studying the MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) -protein serine/ threonine kinases that are activated by ERK or p38 and which phosphorylate eIF4E, a component involved in the initiation of cap-dependent translation. Surprisingly, they found that Mnk1/2 double knockout mice not only exhibited normal cell growth and development despite an absence of eIF4E phosphorylation, but that the tumorigenesis occurring in a mouse model was suppressed by the loss of Mnk1/2. Furthermore, stable knockdown of Mnk1 in a human glioma cell line resulted in dramatically decreased tumor formation when these cells were injected into athymic nude mice. Thus, their data demonstrate an oncogenic role for Mnk1/2 in tumor development, and highlight these molecules as potential anticancer drug targets that could be inactivated with minimal side effects. Clearly, translating such findings to warrant a clinical trial will require many more steps but it shows that despite the complexity of cellular biochemical pathways it may be possible to pin-point specific components that though participating in normal functions can be dispensed with when they become involved in pathological processes. Many other results from clinical trials of rationally designed therapeutics, some disappointing, others successful such as the inhibitor of the ABL tyrosine kinase, imatinib (Gleevec®) and the anti-tumor necrosis factor monoclonals infliximab (Remicade®) and adalimumab (Himura®), could be cited.

So how realistic are the proposals that our increased insight into mast cell biology can be translated to yield therapeutic benefits? To state my own cautious optimism in semi-quantitative terms, I believe that whereas our knowledge can be expected to continue to expand exponentially, the applicability of that knowledge will proceed only linearly and with a rather shallow slope. The enormous expense of clinical trials makes it critical that pre-clinical investigations should be exhaustive, and we must be careful not to let those who pay for our research, whether it be the general public or private investors, be encouraged to believe otherwise.

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1. Ueda T, Sasaki M, Elia AJ et al. Combined deficiency for MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) delays tumor development. Proc Natl Acad Sci USA 2010; 107:13964-13990.

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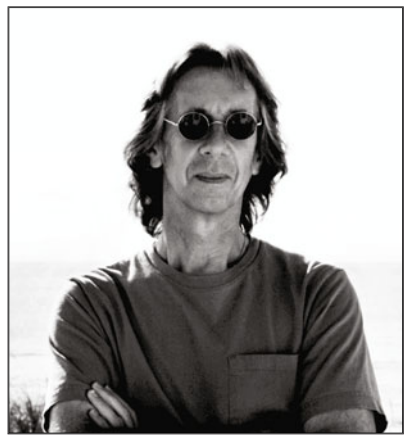

ALASDAIR GILFILLAN, PhD received a BSc from the University of Strathclyde, Glasgow, Scotland and MSc and PhD degrees in Pharmacology from the University of Manchester, England. Following completion of his PhD, he moved to the United States to undertake research in Cell Biology and Biochemistry in the Department of Pediatrics, Yale University Medical School; initially as a Postdoctoral Fellow, then as an Associate Research Scientist. From there, Dr. Gilfillan moved to F. Hoffman-La Roche Ltd (Nutley, New Jersey, USA) where he spent several years conducting preclinical research in the areas of allergy and inflammation. In 1999, he joined the Laboratory of Allergic Diseases (LAD), at the National Institute of Allergy and Infectious Diseases (Bethesda, Maryland, USA), where he currently helps direct the basic research efforts within the Mast Cell Biology Section. Dr. Gilfillan has had a long term interest in mast cell biology, and his current research focuses on the elucidation of signaling mechanisms associated with the activation of mast cells through FcεRI, KIT and other receptors, in health and disease. Dr. Gilfillan is the author of more than 90 scientific articles, and more than 50 abstracts. From 1994-2001, he was on the editorial board of the *Journal of Pharmacology* and Experimental Therapeutics. He currently serves on the editorial boards of *Journal of Immunology*, *Immunology Letters*, *The Open Immunology Journal*, *The Open Allergy Journal*, and the *World Journal of Biological Chemistry*.

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DEAN METCALFE, MD, received a BS from Northern Arizona University and an MD from the University of Tennessee. He completed a residency in Internal Medicine at the University of Michigan and received a MS in Microbiology from the University of Michigan. Dr. Metcalfe was a Fellow in Allergy and Immunology at NIH and a Fellow in Rheumatology and Immunology at the Robert Brigham Hospital and the Harvard Medical School. In 1979, he returned to NIH as a Senior Clinical Investigator. His subsequent career has been devoted to research and public service at the NIH. There he combines clinical and basic research, patient care and administrative duties, as well as scholarly activities in the form of teaching, writing, and speaking on areas related to allergic diseases. In 1995, Dr. Metcalfe was appointed the first Chief of the newly created Laboratory of Allergic Diseases (LAD), NIAID. He is also Chief of the Mast Cell Biology Section/LAD and Associate Director of the NIH Clinical Center Allergy and Immunology Clinical Fellowship Program which is administered through the LAD. Dr. Metcalfe is an author on over 490 scientific papers and 230 abstracts; and an editor on 12 books on mast cell biology, systemic mast cell disorders, food allergy and asthma. He is a past Chairman of the American Board of Allergy and Immunology; a past President of the American Academy of Allergy, Asthma and Immunology; and a past Chairman of the ACGME-RRC for Allergy and Immunology. Dr. Metcalfe has been elected to membership in the American Clinical and Climatological Association, the Collegium Internationale Allergologicum, the American Society for Clinical Investigation, and the American Association of Physicians.

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We thank the chapter authors for their dedication in contributing up to date, outstanding manuscripts for *Mast Cell Biology: Contemporary and Emerging Topics*.

SECTION I

INTRODUCTION

CHAPTER 1

MAST CELL BIOLOGY: Introduction and Overview

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Abstract: In recent years, the field of mast cell biology has expanded well beyond the boundaries of atopic disorders and anaphylaxis, on which it has been historically focused. The biochemical and signaling events responsible for the development and regulation of mast cells has been increasingly studied, aided in large part by novel breakthroughs in laboratory techniques used to study these cells. The result of these studies has been a more comprehensive definition of mast cells that includes added insights to their overall biology as well as the various disease states that can now be traced to defects in mast cells. This introductory chapter outlines and highlights the various topics of mast cell biology that will be discussed in further detail in subsequent chapters.

INTRODUCTION

Mast cells are cells of hematopoietic origin which have gained notoriety over the years for their role as central players in atopic disorders and anaphylaxis. Indeed, it has been in this context that much of the research in this field has been conducted. It is only recently that their role in other aspects of health and disease has been fully appreciated. The manifestations of mast cell-driven disease are considered to be a consequence of an inappropriate activation of mast cell immune responses which have evolved to protect the body against a host of pathogens and perhaps toxins. The biochemical processes regulating mast cell development and mast cell activation have been extensively investigated and comprehensively reviewed in recent years. Hence, rather than reviewing these topics at length, in this work we have opted to focus on the emerging concepts in mast cell biology

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with regards not only to mast cell development and activation, but also on the newly defined roles of mast cells in health and disease. To accomplish this goal, we have solicited contributions from recognized experts in the field of mast cell biology who are focusing on these topics. The scope of this effort cannot be all encompassing and accordingly, not all recent contributions to the field of mast cell biology can be covered. The lack of citation of specific studies thus does not imply that they are of lesser merit or impact.

To set the stage for the more in depth discussions that follow, we begin by presenting a brief overview of mast cell biology in general, in which we indicate those topics that will be elaborated upon in subsequent chapters.

MAST CELL GROWTH, DEVELOPMENT AND SURVIVAL

Mast cells, at least in the human, develop from $CD34^+/CD117^+$ pluripotent progenitor cells originating in the bone marrow.[1] The progression of these cells to fully mature mast cells is dependent on KIT activation which occurs as a consequence of stem cell factor (SCF)-induced KIT dimerization and auto-phosphorylation. Hence, $Kit^{W/W\text{-}v}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice in which surface expression of KIT, or KIT catalytic activity, is defective, have substantially reduced mast cell numbers.[2] Nevertheless, whereas human mast cells in culture require SCF for growth, mouse mast cell growth and expansion from bone marrow progenitors can be maintained by IL-3 in the absence of SCF.[1]

In both the mouse and human, committed bone marrow mast cell progenitors are released into the bloodstream from where they subsequently migrate into the peripheral tissues, during which time they mature and become terminally differentiated under the influence of cytokines within the surrounding milieu.[3] As discussed by Jenny Hallgren and Michael F Gurish in Chapter 2,[4] the migration of mast cell progenitors appears to be controlled in a tissue specific manner. They describe, for example, that basal trafficking of mast cell progenitors into the intestine, a process regulated by dendritic cells expressing the transcriptional regulatory protein, T-bet, requires that $\alpha4\beta7$ integrin and the chemokine receptor, CXCR2, be expressed on the surface of the mast cell progenitors and that MAdCAM-1 and VCAM-1 be expressed on the intestinal endothelium. In contrast, the marked recruitment of committed mast cell progenitors to the lung, observed with the onset of pulmonary allergic inflammation, requires the expression of the $\alpha4\beta7$ and $\alpha4\beta1$ integrins on the mast cell progenitors and associated expression of VCAM-1 on the endothelium, as regulated by CXCR2. Full recruitment however requires activation of CCR2 pathways following binding of CCL2. Such migration of mast cell progenitors from the vasculature into the peripheral tissues is increased under inflammatory conditions, thus providing an explanation for the increased mast cell burden observed at sites of inflammation.[4]

Terminally differentiated, tissue-resident mast cells are long lived, a feature, at least in the human, that is dependent upon the continued presence of SCF. Accordingly, inhibition of KIT catalytic activity by tyrosine kinase inhibitors induces human mast cell apoptosis.[5] As discussed by Maria Ekoff and Gunnar Nilsson in Chapter 4,[6] the Bcl-2 family of proteins are key regulators of such mast cell homeostasis through balancing mast cell survival and apoptosis. They further explain that apoptosis is regulated by both extrinsic pathways and intrinsic pathways which respond to stress from SCF deprivation, DNA damage and other intracellular stimuli and that these pathways involve caspase activation. Extrinsic apoptotic signals are transmitted through death receptors belonging

to the TNF receptor family, such as Fas/CD95R and TRAIL, which have been identified on both murine and human mast cells. However, whereas Fas/CD95R activation has been found to induce apoptosis in murine mast cells, in human mast cells, apoptosis follows TRAIL-R crosslinking.[6]

Ekoff and Nilsson[6] moreover discuss how SCF regulates mast cell survival through inactivation of FOXO3a and subsequent down-regulation of the pro-apoptotic BH3-only protein, Bim, by its ubiquitination and proteasomal degradation following its phosphorylation. They additionally note that studies using Bcl-2 family gene-deficient mice and cells have revealed that the BH3-only protein, Puma, is also critical for the induction of mast cell apoptosis following cytokine deprivation. Under such cytokine deprived-conditions, in wild type cells, both Bim and Puma are upregulated with resulting mast cell apoptosis. The role of the downstream effector proteins Bax and Bak is demonstrated by the fact that their absence abolishes most apoptotic responses induced by BH3-only proteins and by the increased mast cell numbers found in tissues of mice lacking Bax or Bak.[6]

MAST CELL MEDIATORS AND DISEASE

The ability of mast cells to function in both health and disease is dependent on not only the relative abundance of mast cells in tissues, but also on the extent and nature of mediators released. These latter features are respectively dictated by the threshold and magnitude of mast cell activation and by the selective activation of specific signaling pathways controlling the release of individual classes of inflammatory mediators.

Mast cells have the capacity to release a multitude of pro-inflammatory mediators.[3] The immediate response upon mast cell activation to an appropriate stimulus is degranulation; a process characterized by the extrusion of cytoplasmic granule contents into the extracellular space by the process of exocytosis. Although the membrane-proximal signaling processes that allow mast cells to degranulate have been extensively studied and described,[7,8] it is only recently that the detailed mechanics of degranulation and the molecular players regulating this process have been investigated. In Chapter 7, Ulrich Blank[9] discusses recent work on defining the roles of specific proteins which participate in the regulation of membrane fusion during exocytosis and the connection of the fusion machinery with early signaling events initiated upon mast cell activation.

Mast cell granules are rich in bioactive amines, proteoglycans and proteases.[3] Clinically, the most important bioactive amine present in the granules is histamine. The role of histamine in human disease is well established. For example, histamine, along with PGD_2 and LTC_4, induces bronchoconstriction, mucus secretion and mucosal edema and thus contributes to the symptoms of asthma.[10,11] As described by Elena Moiseeva and Peter Bradding in Chapter 13,[12] the relative rate of mediator release from human lung mast cells in vitro is histamine > PGD_2 > LTC_4 with one-half maximal release occurring at 2, 5 and 10 minutes respectively and this appears to parallel in vivo release in the human lung. Serotonin (5-hydroxytryptamine) is also relatively abundant in mouse mast cell granules, but it only represents a minor component of the human mast cell granules[13] and to date has not been shown to be a relevant mediator in human mast cell-driven disease.

Mast cell granules are particularly rich in proteases. These proteases are the major proteins present in mast cells; representing up to 50% of the total protein content of the granule.[14] The major protease present in human mast cells is β-tryptase.[15] Chymase is also

present in substantial amounts in particular sub-populations of mast cells and the relative abundance of tryptase and chymase in human mast cells has been employed to define particular mast cell phenotypes.[16] These granule-associated proteases are complexed to proteoglycans. One example is heparin which helps to stabilize the proteases and influence function.[14] As discussed by George Caughey in Chapter 12,[17] mast cell proteases have both detrimental and beneficial actions. In this respect, although proteases have been implicated in inflammatory process including those associated with allergic inflammation, they may be protective and even anti-inflammatory in some settings.

Activation of mast cells results in the rapid generation of lipid-derived inflammatory mediators.[18] Both membrane-associated phospholipids and sphingolipids provide substrate sources for lipid derived pro-inflammatory mediators. Through the action of cytosolic phospholipase (cPL)A_2, membrane phospholipids, particularly arachidonyl-containing phosphatidylcholine and, to a lesser extent, phosphatidylethanolamine, are hydrolyzed at the sn_2 position to yield free arachidonic acid which can be subsequently metabolized by the respective actions of cyclo-oxygenase and 5-lipoxygenase to produce the eicosanoids, prostaglandin (PG)D_2 and leukotriene (LT)C_4.[19] PLA_2 activation also induces deacylation of the membrane-associated phospholipid, alkylacylglycerophosphorylcholine to form lyso-platelet activating factor (lyso-PAF) which is subsequently reacetylated by the action of acetylCoA:lyso-PAF acetyltransferase to produce PAF which has been implicated in systemic anaphylaxis.[20] As discussed by Ana Olivera and Juan Rivera in Chapter 8,[21] the sphingolipid-derived mediator sphingosine 1-phosphate, generated by mast cells following activation, is capable of inducing biological responses in mast cells and surrounding tissues. It, thus, may play a role in the regulation of diverse biological systems, including the immune system. Such responses can be mediated by both receptor-mediated and receptor-independent pathways.[22]

Following these initial events, which happen within minutes of mast cell activation, there occurs an enhancement of gene expression leading to the generation of an array of cytokines including GM-CSF, TNFα, IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13, chemokines including CCL2, CCL3, CCL5 and CXCL8 and growth factors such as SCF, FGF, VEGF and angiogenin.[23-29] As discussed by Christopher Shelburne and Soman Abraham in Chapter 10,[30] by Mindy Tsai, Michele Grimbaldeston, and Stephen Galli in Chapter 11,[2] by Elena Moiseeva and Peter Bradding in Chapter 13,[12] and by Domenico Ribatti and Enrico Crivellato in Chapter 14,[31] these mediators can have a profound influence on surrounding tissues and can result in a wide array of cellular responses, including induction of immune and inflammatory responses, cellular hyperplasia, angiogenesis and tumorigenesis.

MAST CELL ACTIVATION

Mast cells express a multiplicity of cell surface receptors which have the capacity to impact mast cell responses through the regulation of proliferation, migration and activation. However, the two major receptors responsible for regulating mast cell functions are KIT and the high affinity receptor for IgE, FcεRI.[32,33]

Allergen-induced mast cell activation occurs as a consequence of FcεRI aggregation through antigen-dependent cross-linking of antigen-specific IgE molecules bound to the FcεRI.[33] However, as described by Jun-ichi Kashiwakura, Iris M. Otani and Toshiaki Kawakami in Chapter 3,[34] recent studies have provided evidence that occupancy of FcεRI with IgE, in the absence of antigen, will provoke mast cell responses. These responses,

which are attributed to "monomeric" IgE, include induction of chemokine production, mast cell chemotaxis and the prevention of apoptosis.[35] Regardless, it is clear that FcεRI aggregation is required to elicit the necessary signaling events for the full extent and range of antigen-induced inflammatory mediator release.[36]

There is an increasing appreciation that such mast cell activation can be up-regulated or down-regulated through other receptors expressed on mast cells and that many of these receptors can by themselves activate mast cells.[37,38] However, the role of these receptors in regulating mast cell function in a physiological setting remains unclear. As described elsewhere in this overview, KIT is an essential growth and anti-apoptotic factor for the generation of mast cells from progenitors and for the subsequent survival of mature, tissue-resident mast cells. SCF-dependent KIT activation, however, also enhances antigen-mediated mast cell degranulation and cytokine production.[39,40] Similarly, signaling events initiated by KIT and FcεRI synergistically interact to markedly enhance mast cell chemotaxis.[41] These responses are also observed with a number of GPCR agonists including PGE_2 and adenosine.[41]

The demonstration that a wide array of bacterial products, viral products, parasite products, toxins, Toll-Like Receptor (TLR) agonists and host defense products also induce activation of mast cells has led to the realization that mast cells are an important component of innate immunity as outlined by Christopher Shelburne and Soman Abraham in Chapter 10.[30] The consensus of data, however, suggests that unlike antigen-, KIT- and GPCR-mediated responses, those elicited through TLRs interacting with viral, bacterial and parasitic products, are likely restricted to induction of cytokine and chemokine production. As with KIT and GPCRs, such responses are synergistically enhanced when mast cells are co-activated through the TLRs and FcεRI.[42] This differential regulation of specific categories of mediators by alternative classes of mediators directly reflects their selective utilization of specific adaptor molecules for relaying signals. In this respect, unlike the transduction pathways induced by FcεRI, GPCRs and KIT, signals relayed by the major adaptor molecule for the TLRs, MyD88, do not lead to enhanced intracellular calcium levels, a necessary signal for mast cell degranulation.[42] It is of interest that another MyD88-linked receptor, the IL-33 receptor, enhances cytokine production in mouse and human mast cells,[43] while having minimal effect on degranulation.[44]

MAST CELL SIGNALING

Identification and characterization of the molecular components of the signaling pathways that regulate mast cell responses has been an active area of research over the past two decades and much is now known regarding these processes. These events have been extensively reviewed in a series of articles to which the readers are referred to for further details.[7,8,33,37,45] Here we only present a brief overview of this topic, primarily focusing on FcεRI.

Signaling initiated by FcεRI aggregation leads to the intracellular assembly of a membrane-associated receptor-signaling complex. FcεRI comprises an IgE-binding α subunit, a tetramembrane-spanning β subunit and homodimeric disulphide-linked γ chain tandem subunit.[33] Both the β and γ chains contribute to the transfer of signals across the membrane. However, whereas the γ chain is essential for transducing signals required for mast cell activation, the role of the β chain appears to be to amplify the signals induced by the γ chains.[33]

Although it is clear that the subsequent receptor-proximal signaling events involve a series of protein phosphorylation events and protein-protein interactions,[7] the precise locations where interactions take place in the context of the three dimensional structure of the cell and the real time kinetics of these processes, are relatively unknown. However, as described by Bridget Wilson, Janet Oliver and Diane Lidke in Chapter 6,[46] a concept that has emerged during the past decade centers on the localization of early signal transduction events to specialized membrane microdomains. As described, electron microscopy and other approaches have revealed that, following aggregation, FcεRI and specific signaling molecules localize to discrete membrane "patches" which may represent morphological evidence of lipid rafts. These rafts, otherwise termed glycolipid-enriched domains, have been described based on their physical properties and chemical composition.[47] However, these studies also reveal that these regions are not uniform and that not all signaling molecules localize to the same domains.[48] It thus remains to be determined how the signaling molecules restricted to the discretely separate membrane domains interact. Nevertheless, biochemical approaches have provided evidence that, following antigen challenge, aggregated FcεRI coalesce with the putative lipid rafts, where they interact with key signaling molecules to initiate the cascades required for downstream mediator release.[49,50]

The initial key receptor-signaling protein interactions required for mediator release follow phosphorylation of immunoglobulin receptor activation motifs (ITAMs) contained within the β and γ chain cytosolic domains as mediated by the Src family tyrosine kinase Lyn; and subsequent recruitment, phosphorylation and activation of Syk tyrosine kinase. There still remains an enigma, however, regarding the role of Lyn in these initial steps. Although it is assumed that Lyn is responsible for the initial phosphorylation of the FcεRI β and γ chain ITAMs and thus would be essential for downstream mediator release, data from *lyn*$^{-/-}$ mice and mast cells derived from the bone marrow of these mice, suggest that Lyn is dispensable for mast cell activation. Indeed, depending on the genetic background of the mice, hyperactivation of mast cells following antigen challenge has been observed in the absence of Lyn.[51] This may be a reflection of redundancy in the roles of individual Src kinases in the initial stages of mast cell activation, as other Src kinases including Fyn,[52] Fgr[53] and Hck[54] have been documented to also contribute to mast cell activation.

Following these initial signaling events, a bifurcation in the pathways takes place allowing the activation of two major signaling enzymes; PLCγ and phosphoinositide 3-kinase (PI3K). However, intercommunication between these pathways likely occurs. These events are coordinated by specific protein-protein interactions and subsequent assembly of a macromolecular signaling complex through particular binding motifs contained within transmembrane and cytosolic adaptor molecules. PLCγ is recruited into the signaling complex through its direct binding to the transmembrane adaptor molecule LAT following its phosphorylation by Syk; an interaction stabilized through secondary indirect binding via the cytosolic adaptor molecules Gads and SLP76,[55] whereas PI3K is recruited to the receptor-signaling complex via the Fyn- and/or Syk-dependent phosphorylation of Gab2.[52,56,57] There is also evidence to suggest that PLCγ_1 additionally binds indirectly to the LAT-related transmembrane adaptor LAT2/NTAL/LAB.[58] KIT also utilizes PLCγ for downstream signaling. However, in contrast to the FcεRI, KIT contains a recognized PLCγ-binding motif in its cytosolic domain. As a consequence, following KIT ligation and phosphorylation, KIT directly binds and activates PLCγ_1.[59] Although the GPCRs that influence mast cell mediator release do not activate PLCγ they do activate the

functionally related PLCβ through GPCR βγ subunits. TLRs, however, activate neither PLCγ nor PLCβ, thus explaining their lack of effect on mast cell degranulation.

Through the hydrolysis of phosphoinositide 4,5, bisphosphate (PIP_2) and the consequential production of inositol trisphosphate (IP_3) and diacylglycerol, PLC activation leads respectively to an increase in cytosolic calcium levels and activation of protein kinase C (PKC).[60] IP_3 induces elevated cytosolic calcium concentrations by receptor-mediated liberation of calcium from the endoplasmic reticulum (ER).[61] The emptying of the ER stores of calcium in this manner triggers a secondary, more pronounced, calcium signal through store operated calcium entry (SOCE) from extracellular sources. As described by Hong-Tao Ma and Michael Beaven in Chapter 5,[62] recent studies have begun to identify the molecular players and interactions that regulate this latter process. In this respect, the sensor that detects the emptying of calcium from the ER has been identified as stromal interacting molecule-1 (STIM1)[63,64] and the corresponding calcium channel on the cell membrane allowing SOCE as ORAI1.[65,66] Other calcium channels, termed transient receptor potential canonical (TRPC) channels, also likely contribute to SOCE. However, the precise manner in which TRPC channels interact with ORAI and STIM has yet to be determined. The calcium signal is eventually terminated upon re-uptake of calcium and replenishment of the calcium stores within the ER via an ATP-dependent sarco/ER Ca^{2+} ATPase (SERCA) pump; and removal of excess cytosolic calcium across the cell membrane by TRPMV4-mediated depolarization of the cell membrane, through Na^{+}/Ca^{2+} exchange, or through ATP-dependent plasma membrane Ca^{2+} ATPase (PMCA) pump.[67]

PI3K phosphorylates PIP_2 at the 3′ position, thereby generating phosphoinositide 3,4,5 trisphosphate (PIP_3).[68] This provides membrane docking sites for PH domain-containing signaling proteins, for example, PLCγ, Btk, PDK1 and AKT.[68] PI3K is a family of heterodimeric complexes comprising a catalytic and an adaptor subunit. Both KIT and the FcεRI signal through the PI3Kδ family member,[69] whereas GPCRs signal through the PI3Kγ family member.[70] PI3K is indispensible for KIT mediated mast cell responses and for FcεRI-mediated cytokine production. This likely reflects the control of multiple downstream signaling cascades by PI3K, including those regulated by the MAP kinases, ERK1/2, p36 and JNK, mTORC1 and mTORC2, and GSK3β, which contribute to the regulation of gene transcription and or chemotactic responses.[70] PI3K also contributes to mast cell degranulation and the enhancement of this response by KIT. However, a fraction of FcεRI-mediated degranulation is refractory to the influence of PI3K.[71] This has led to the suggestion that PI3K contributes to degranulation through the recruitment of Btk and subsequent amplification/maintenance of PLCγ-mediated calcium signals required for degranulation.[72,37]

The status of mast cell activation at any point in time is in reality a balance between the signaling pathways discussed above and those that down-regulate these processes. As discussed by Laila Karra and Francesca Levi-Schaffer in Chapter 9,[73] this fine balance is essential to avoid inappropriate or exaggerated mast cell-mediated responses. A variety of receptors are expressed on mast cells that posses the capacity to downregulate and thus buffer, receptor-induced mast cell activation.[74] These receptors are characterized by a conserved immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytosolic domains which, when phosphorylated, recruits the protein phosphatase SHP-1 and the lipid phosphatases SHIP1 and SHIP2. Whereas SHP-1 reverses protein-protein interactions mediated by tyrosine kinases, SHIP1 and 2 dephosphorylate PIP_3, thus reversing the association of essential PH domain-containing signaling proteins with the membrane-associated signaling complex.[74] As discussed,[73] disruption of the balance

between these inhibitory pathways and those that lead to up-regulation of mast cell responses, could result in inappropriate activation of mast cells leading to disease conditions.

CONCLUSION

The understanding of mast cell biology has dramatically increased over the past two decades, largely due to three major developments. The first of these was the discovery of growth factors in both mouse and man that allowed the culture of mast cells from marrow and peripheral blood. The second major breakthrough was the realization that mast cells could be cultured from the marrow of mice with either known genetic defects or mice where specific gene expression was directly manipulated. A corollary was the development of approaches where mast cells could be used to replete the mast cell compartment in mice deficient in mast cells. This latter approach allowed the dissection of the role of mast cells in diseases states, at least in the mouse. Finally, the application of genomics and proteomics applied to the study of human mast cells has allowed the association of mast cell defects to human disease states. One major example of this was the association of mutations in KIT with human mastocytosis.

The work summarized in this chapter, which reflects the contributions of the individual chapter authors, well illustrates how mast cell biology has advanced and how these discoveries have facilitated the recognition and acceptance of mast cells as critical to both innate and acquired human immune responses.

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SECTION II

MAST CELL DEVELOPMENT AND HOMEOSTASIS

CHAPTER 2

MAST CELL PROGENITOR TRAFFICKING AND MATURATION

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Abstract: Mast cells are derived from the hematopoietic progenitors found in bone marrow and spleen. Committed mast cell progenitors are rare in bone marrow suggesting they are rapidly released into the blood where they circulate and move out into the peripheral tissues. This migration is controlled in a tissue specific manner. Basal trafficking to the intestine requires expression of α4β7 integrin and the chemokine receptor CXCR2 by the mast cell progenitors and expression of MAdCAM-1 and VCAM-1 in the intestinal endothelium; and is also controlled by dendritic cells expressing the transcriptional regulatory protein T-bet. None of these play a role in basal trafficking to the lung. With the induction of allergic inflammation in the lung, there is marked recruitment of committed mast cell progenitors to lung and these cells must express α4β7 and α4β1 integrins. Within the lung there is a requirement for expression of VCAM-1 on the endothelium that is regulated by CXCR2, also expressed on the endothelium. There is a further requirement for expression of the CCR2/CCL2 pathways for full recruitment of the mast cell progenitors to the antigen-inflamed lung.

INTRODUCTION

Mature mast cells are found in many connective tissues where they are rare long-lived cells. These constitutive and connective tissue-associated mast cells have a restricted phenotype based on mediator production and secretory granule proteases that varies somewhat from tissue to tissue. An entirely different phenotype, prominent after a nematode infection, was first identified in the mouse and rat associated with the intestinal mucosa. This rodent mucosal phenotype also shows some variability in granule content.

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Nonetheless, these findings led to our current paradigm that there are two classes of mast cells, the connective tissue and the mucosal phenotypes. In general, two broad classes of mast cells also have been identified in the human based predominantly on differences in secretory granule protease expression. All phenotypes appear to be derived from a common mast cell progenitor with the final phenotype determined by micro-environmental factors such as cytokines or perhaps by interactions with the extracellular matrix.[1-3] In this chapter we address the issues of how mast cells arise and how they get to their diverse sites of residency. Most of the discussion will be focused on studies conducted in the mouse but wherever possible, differences and similarities in the human will be mentioned.

MAST CELL DEVELOPMENT

The seminal studies by Kitamura and colleagues in the late 1970s showing reconstitution of mast cells in mast cell-deficient mice by the adoptive transfer of wild type bone marrow indicated these cells could be derived from the bone marrow and thus were of hematopoietic origin.[4,5] Then, in the early 1980s, the ability to grow cultured mast cells from murine bone marrow using Interleukin (IL)-3 was discovered.[6,7] This propelled the studies on understanding the developmental pathway and the observation that the immature mast cell progenitors could be found in peripheral tissues such as in the intestine.[8-10] In the last few years, a number of studies by Akashi and colleagues as well as others have defined several of the intermediate steps in what has sometimes been termed the classical model of hematopoiesis.[11-14] These define the development of the mast cell in mouse bone marrow occurring along the myeloid pathway. In this model, the initial fate decision made by the hematopoietic stem cell is to commit to the myeloid or lymphoid lineage. This first step in myeloid development is characterized by the down regulation of the Sca-1 antigen. This intermediate has been termed the Common Myeloid Progenitor (CMP) and is distinguished from the Common Lymphoid Progenitor by expression of IL-7R by the latter cells. The CMP intermediate can give rise to either the Megakaryocyte-Erythrocyte Progenitor or to the next step in myeloid development, the Granulocyte Macrophage Progenitor (GMP) which is distinguished by the up regulation in expression of the low affinity IgG receptors, FcγRII/III (identified by the 2.4G2 mAb). The GMP can give rise to macrophages, eosinophils, neutrophils or the novel Basophil-Mast Cell Progenitor (BMCP) first identified in the spleens of C57BL/6 mice. These BMCP, were only found in the spleen of this mouse strain and could be identified as a KIT^+, $Fc\gamma RII/III^+$, $\beta 7\ integrin^{hi}$, $Fc\varepsilon RI^-$ cells, that only gave rise to mast cells or basophils in culture despite using cytokines that give rise to all the myeloid cells after culture of bone marrow cells. Furthermore, transfer of these BMCP into mast cell-deficient mice led to the appearance of mast cells in the spleen and peritoneal cavity, demonstrating their capacity was not limited to in vitro differentiation.[12] The isolation of the CMP, GMP and BMCP; and the ability of these cells to differentiate into basophils in culture have been confirmed by others.[15]

A second mast cell differentiation pathway in adult mice has also been described in which the cells are derived from the multipotential progenitor.[16] In these experiments, the authors isolated the mast cell progenitors as lineage negative (including lacking the high affinity IgE receptor, FcεRI), $CD27^-$, $\beta 7\ integrin^+$, $IL33R\ (T1/ST2)^+$. This fraction, as with the BMCP, was able to restore mast cells but not other lineages after transfer into mast cell-deficient mice indicating their lineage potential was restricted. Based on these

finding the authors proposed an alternate model of differentiation for the mast cell as occurring via a direct commitment from the multipotential progenitor. This development scheme is more in keeping with the alternative model of hematopoietic development suggested by Kawamoto and others.[17-19] This model was first developed using fetal liver-derived cells but has been extended to adult hematopoiesis as well. It suggests that myeloid developmental potential is retained beyond the first commitment step. In fact, in this model, myeloid potential is retained even past the differentiation between B and T-lymphocyte commitment. Support for this comes from many investigations both in vitro and in vivo based on findings such as the demonstration that up to 30% of the macrophages in the thymus are derived from the early T cell progenitors migrating into the thymus from the bone marrow.[17]

In either scheme, it is thought the committed mast cell progenitor is released into the blood stream where it migrates to various tissues as discussed further below. While there has been little investigation of the blood-borne mast cell progenitor in adult mice, in 1996, Rodewald et al demonstrated for the first time a committed mast cell progenitor in fetal mouse blood.[20] This cell, identified as KIT^+, Thy-1^{lo} was unusually prevalent at fetal day 15.5 allowing its identification. The numbers subsequently dropped down suggesting the cells were perhaps seeding tissues at a particular stage in development. Given the difficulty in restoring normal mast cell numbers in many tissues, it may be that there is an important developmental period where mast cells seed tissue such as the skin and provide the indigenous cell population with little turn over.[21]

The surprising ease with which murine bone marrow gave rise to mast cell lines in vitro was not initially reproducible with human hematopoietic progenitors. It awaited our understanding of the importance of stem cell factor (SCF, also called KIT ligand, KITl) in the growth and differentiation of mast cells before routine in vitro culture of mast cells from human cord blood was established.[22-24] This is now an established technique for studying human mast cells in vitro and has been used in many studies such as to demonstrate that the human mast cell progenitor that can be identified in peripheral blood, is closely related to macrophages in development and is increased in the blood of asthmatics.[25-27]

The critical role of SCF in mast cell development was first suggested by the discovery of mutations in the *kit* locus, first identified as the *W* locus, which resulted in a mast cell deficiency of varying degrees. In the absence of full KIT signaling, mast cell maturation is truncated and, depending on the mutation, results in partial or almost complete deficiency of mast cells in the peripheral tissues. The ability to generate early myeloid progenitors is not eliminated, however, leaving the ability to develop mast cells in culture via IL-3 intact.[28] Nonetheless, the influence of SCF on development is dramatic, driving maturation of the cells toward a connective tissue phenotype and sustaining the cells both in vitro and in vivo.[29-31] Unfortunately, as this receptor is involved in myelopoiesis, gametogenesis and melanogenesis, loss of this receptor results in more than a simple mast cell deficiency. Among other affects, there are decreased numbers of basophils in the bone marrow and BMCP in the spleen; and it also results in a neutropenia which can influence results based on responses involving these two cells,[12,32] Thus, caution is necessary in interpreting the results obtained using KIT- or SCF-deficient mice.

As noted above, Rodewald et al provided one of the first phenotypic definitions of a committed mast cell progenitor. This Thy-1^{lo}, KIT^+ fetal blood cell responded only to SCF and IL-3 together in vitro and lacked any potential for becoming macrophages, granulocytes, erythrocytes or B or T-lymphocytes in several different assays, but gave rise to mast cells at a high frequency. To date a phenotypic definition of committed mast

cell progenitors in adult mouse (or human) blood has not been completed, although we have identified committed mast cell progenitors in both the mouse intestine and lung as lineage$^-$, CD45$^+$, CD34$^+$, FcεRI$^+$, β7 integrin^{hi} cells and a c-kit$^+$, CD34$^+$ macrophage/mast cell progenitor is found in human peripheral blood.[12,25,33] Of note is the continued expression of the CD34 marker of murine mast cells throughout their maturation, although this is usually a marker of early hematopoietic progenitors.[34,35]

BASAL HOMING OF MAST CELL PROGENITORS

Most studies of migration and trafficking have focused on movement into the small intestine or peritoneal cavity. This is because of the relatively large number of committed mast cell progenitors or easily obtainable mature mast cells, respectively, found at these peripheral tissue sites in rodents. The pool of committed mast cell progenitors in the intestine is greater than that found in bone marrow (among mononuclear cells isolated from the tissues), although there are many more mononuclear cells and therefore cells within this lineage in the bone marrow. This large intestinal pool of committed mast cell progenitors facilitated the initial studies identifying these cells using clonogenic assays with IL-3 alone or in combination with SCF.[8,10,36-38] It also facilitated our investigations on the molecules critical to establishing and maintaining this pool. The appreciation of the immature mast cell progenitor was a direct consequence of the early studies highlighting the ability of these cells to be expanded and maintained with IL-3. This led to studies by several labs showing the identification of mast cell progenitors in blood, lung and intestine.[8,36,39] While the early studies used a clonogenic assay that did not distinguish early precursors from the committed mast cell progenitors, the assay did allow the identification of lineage-committed cells and eventually led to the conclusion that a population of committed mast cell progenitors is found in bone marrow, spleen, lung and intestine.[12,16,33]

The identification of β7 integrins on rodent and human mast cells suggested a role for one or both of the β7 integrins, α4β7 or αEβ7 in homing of mast cells to the intestine based on their involvement in the homing of lymphocytes to the small intestine and associated lymphatic structures.[40-43] To test this hypothesis, we used both null strains and blocking antibodies directed against the various integrin chains. These techniques further allowed us to define the critical integrin, the important endothelial ligands, a chemokine receptor and a role for dendritic cells in the establishing and maintaining of the large intestinal pool of committed mast cell progenitors.

Using the β7 integrin null mice, we found a profound deficit in both mast cell progenitors and, as shown by others as well, a deficit in the mature cells in the small intestine.[37,44] The deficit in mature mast cells was in both populations located in the submucosal connective tissue and in the mucosa of the intestine. This lack of both phenotypes in the absence of progenitors supported the paradigm that a single committed progenitor gives rise to all phenotypes of mature mast cells. In order to address the issue of which of the β7 integrin molecules was critical in controlling ingress, we established a protocol to allow the use of antibody blocking. To do this as, we took advantage of the observation made a number of years earlier by Kitamura and colleagues, that the progenitors are very sensitive to irradiation.[45] This allowed us to eliminate the intestinal pool of mast cell progenitors and then study its reconstitution over the course of the next 2 weeks. We could influence the mast cell progenitor reconstitution via the administration of antibodies to the different

integrins. Using anti-α4, anti-β1, anti-β7, anti-αE and anti-α4β7 integrin antibodies, the relevant integrin was defined as α4β7 and not α4β1 or αEβ7. Although after entry, the intestinal-localized mast cell progenitors down regulate expression of α4β7 and up regulate expression αEβ7.[46]

As the α4β7 integrin molecule can bind to both the mucosal addressin cellular adhesion molecule (MAdCAM)-1 or vascular cell adhesion molecule (VCAM)-1, both of which are expressed within the intestine, we evaluated the involvement of each cellular adhesion molecule. Using both null mice lacking VCAM-1 or antibody blocking directed to MAdCAM-1 or VCAM-1, both molecules were showed to be required for the establishment of the intestinal pool of mast cell progenitors. We also evaluated various chemokine receptor null mice looking for specific chemokine pathways involved in this process. We could find no evidence of any role for CCR2, CCR3, or CCR5, while significant inhibition was observed in CXCR2 null mice.[38] This IL-8 receptor is expressed on both circulating leukocytes and on endothelium, suggesting it could be affecting the trafficking either through effects on the circulating mast cell progenitor or on the vascular endothelium.[47] Adapting the techniques of sublethal irradiation followed by bone marrow reconstitution of wild type mice that we used for antibody blocking, we reconstituted the various mice with either wild type or CXCR2-deficient bone marrow. These studies demonstrated reduced reconstitution in the wild type mice given CXCR2 null bone marrow, indicating that the circulating progenitor needs to express CXCR2. Further investigation also noted a role for CXCR2 expression by the host, as reconstitution of CXCR2 null mice with wild type bone marrow also resulted in fewer intestinal mast cell progenitors when compared to wild type mice reconstituted with wild type bone marrow in parallel.[46] These findings are summarized in Figure 1.

The process of reconstitution of the pool of mast cell progenitors in the intestine occurred fairly rapidly after sublethal irradiation and reconstitution with wild type bone marrow, achieving about 50% of the normal frequency by one week and almost complete reconstitution by two weeks.[37] This rapid replenishment of the pool suggested that this could be an innate process ongoing in normal mice. Confirmation of this hypothesis came with the treatment of normal BALB/c mice with an antibody directed to either the α4β7 integrin or to the CXCR2 receptor. In both cases, administration of the antibodies every other day for about one week resulted in a ~75% depletion of the pool of MCp.[38] Since the cells down regulated α4β7 integrin expression and up regulated αEβ7 integrin expression after entering the intestine, the depletion of the pool was a consequence of inhibition of the transmigrating cells attempting to leave the circulation rather that a direct effect on progenitors in the intestines. Support for this interpretation was obtained by monitoring the pool of mast cell progenitors in the lung. This pool is not dependent on either α4 integrins or expression of the CXCR2 receptor; and the size of this pool as well as that in spleen increased when mice were given anti-α4 integrin, suggesting an increase in number of circulating mast cell progenitors as a consequence of preventing them from moving into the intestine. Furthermore, the remarkable depletion of almost 80% after 6 days of treatment with the blocking monoclonal antibody of the pool suggested a half-life for the cells in the intestine of about 4 days.

The innate nature of the basal homing of mast cell progenitors to the intestine was first suggested by Guy-Grand et al in their studies of this process.[10] Using germ free mice, they found there was no diminution in the size of progenitor pool in the absence of intestinal flora. Furthermore, the size of intestinal pool was unaltered in athymic mice which lack mature T cells. We pursued this further using mice lacking the recombinase activating

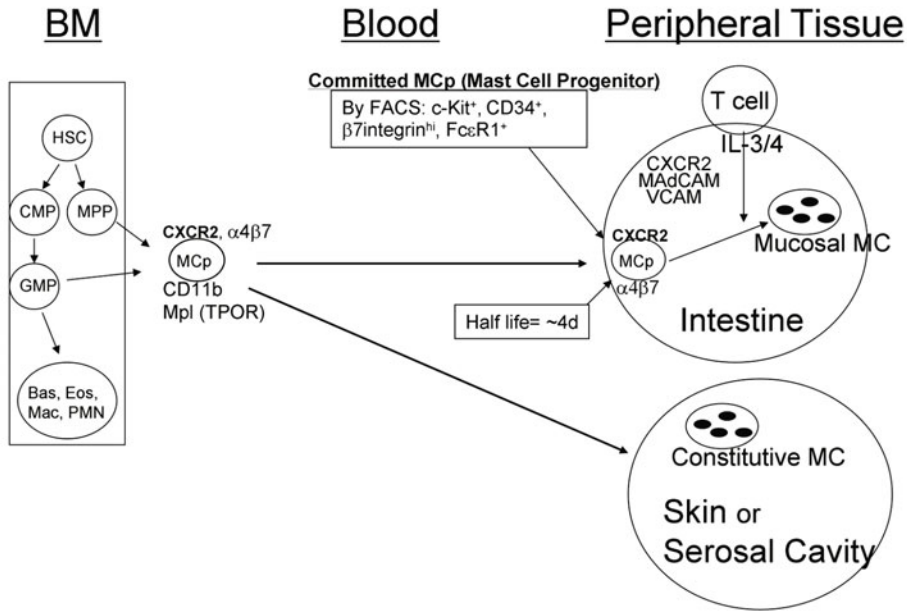

Figure 1. Development of committed mast cell progenitors (MCp) in the bone marrow (BM) from the hematopoietic stem cell (HSC) and the molecules identified as playing a role in the trafficking of the these cells to the intestine and peritoneal cavity under basal, non-inflamed conditions. Two different developmental pathways have been proposed leading to the production of committed mast cell progenitors. These cells then move out to peripheral tissues via the circulation and migrate into the specific tissue sites such as the intestine and serosal cavity under the control of specific molecules such as the α4 integrins and various chemokine pathways. A role for the C3b and TPO receptors has been shown to affect mast cell numbers in the skin and peritoneal cavity. See text for details.

gene (RAG)-2 and the common IL-R gamma chain (common for IL-2, IL-4, IL-7, IL-9 and IL-15 receptors). These double deficient mice lack all mature lymphocytes including natural killer, T, B and natural killer T cells.[48] As in athymic mice, mast cell progenitors homed to the intestine in normal numbers in the absence of all of these other cells types. This was also not a strain specific phenomenon, as both C57BL/6 and BALB/c mice had normal numbers of intestinal mast cell progenitors in the absence of T cells.[37] Given this lack of dependence on the adaptive immune system, we had assumed this was an innate process not dependent on other cell types. Thus, it was surprising to find that in mice lacking the T helper Type 1 master developmental regulator, the transcription factor T-bet (Tbx21), there were decreased numbers of mast cell progenitors in both the lung and intestine of both C57BL/6 and BALB/c mice. This also resulted in fewer numbers of mature intestinal mast cells as expected from previous studies.[49] These results indicated an innate cell type must be involved and affected developmentally by the loss of T-bet. Yet there was no evidence of expression within the mast cell lineage, despite noting that T-bet null mast cells showed decreased adhesion to VCAM-1 under flow. The demonstration that dendritic cells also expressed T-bet and that loss of this protein had an effect on their role in an arthritis model led us to consider whether this cell type played some role in the formation of the intestinal pool of mast cell progenitors.[50] Unexpectedly, the transfer of bone marrow-derived dendritic cells from wild type mice to wild type or

T-bet null mice restored the pool of intestinal mast cell progenitors to similar levels in both strains. Thus, while there is no role for lymphocytes in the trafficking of mast cell progenitors to the lung or intestine, dendritic cell functions or development controlled by T-bet play a critical role in the trafficking of these mast cell progenitors to the intestine and development of mature mast cells in this tissue. In subsequent work, we have repeated these experiments, evaluating the much smaller pool of mast cell progenitors in the lung and found no reconstitution of the pulmonary pool in T-bet null mice by wild type bone marrow-derived dendritic cells (Moravia et al, unpublished observations). Thus, while these studies established a critical role for T-bet$^+$ dendritic cells in controlling the numbers of mast cells in the intestine, this is not the case for the lung. It is remarkable that, as of this time, very little is known about what controls the basal pool of mast cell progenitors in the lung.

Other studies have also implicated a role for CD11b and the TPO receptor Mpl on maturation and movement of the mast cell progenitors into the peritoneal cavity and skin. Thus, reduced numbers of mast cells were found in the peritoneal cavity and dorsal skin in CD11b-deficient mice.[51] However, there was no defect in the number of mast cell progenitors noted in bone marrow or intestine, suggesting that the loss of this C3b receptor plays a role in maturation or survival of the cells at these sites and does not influence trafficking of the cells. In contrast, because thrombopoietin (TPO) has been shown to increase human mast cell colonies in vitro and increase apoptosis of murine mast cells both in vitro and in vivo, TPO receptor (Mpl) null mice were evaluated for number of mast cell progenitors and mature mast cell in various sites. Using the Chen et al definition of bone marrow mast cell progenitors as FcεRI$^-$, CD27$^-$, β7 integrin$^+$ and IL33R$^+$, loss of Mpl resulted in decreased numbers of mast cell progenitors in the spleen and bone marrow, but increased numbers of mature mast cells based on histological staining and fluorescent staining for KIT and FcεRI.[16,52,53] There were also increased numbers of mast cells in the spleen, bone marrow, skin, stomach and peritoneal cavity, suggesting that TPO via its receptor plays an important role in controlling the number of mast cell in several different sites.

RECRUITMENT OF MAST CELL PROGENITORS

Mast cell numbers increase significantly in tissues during inflammatory conditions such as in the joints with arthritis or in the lungs of patients with allergic asthma.[54-56] In asthmatics, this is accompanied by an increase in circulating progenitor cells as assessed in a clonogenic assay.[27] In mice, the normal number of mast cells in the airways of the common wild type strains C57BL/6 and BALB/c is relatively low, but is significantly increased after the induction of allergic airway inflammation.[57,58] This increase may be due to either division of the few mature mast cells in situ or recruitment and maturation of mast cell progenitors in the lung, or both. Because of the constitutive trafficking occurring to maintain the intestinal progenitor pool and previous studies suggesting recruitment to the intestine after helminth infection in mice,[10,59] we hypothesized that the increment in mast cells reflects recruitment of mast cell progenitors via transendothelial migration from the blood to the lung. We expected this to be an active process that is induced during the inflammation and is inhibited when the stimuli is stopped involving specific pathways such as found for basal trafficking to the intestine. To test this, both BALB/c and C57BL/6 mice were

immunized systemically with ovalbumin (OVA) adsorbed to alum on day 0 and day 7, followed by three consecutive daily OVA aerosol challenges starting 10 days later. Both strains have significantly more mast cell progenitors in the lung after challenge, with a magnitude of ~ 28 fold increase in total number of pulmonary mast cell progenitors in BALB/c mice and ~ 10 fold increase in C57BL/6 mice.[60] As reported by others, the histological assessment of inflammation demonstrated that similar OVA treatments did not induce similar degrees of inflammation with C57BL7/6 mice, showing more modest inflammation than in BALB/c.[61] The magnitude of the increase in such a short period of time after the initial antigen challenge indicted that recruitment rather than expansion of the small numbers of resident progenitors was the most likely explanation and led to the investigation of specific molecules and cells that controlled the process.

During OVA-induced lung inflammation, VCAM-1 expression on the lung endothelium is up regulated.[62] Using endothelial VCAM-1 knock out mice and anti-VCAM-1 treatment before each OVA-aerosol challenge, we showed that mast cell progenitor recruitment is critically dependent on VCAM-1 expression on the endothelium in the lung. In contrast to the homing of mast cell progenitors to intestine, there was no role for MAdCAM-1 in the recruitment to lung, consistent with reports that MAdCAM-1 is not expressed in the lung endothelium.[63,64] As both $\alpha4\beta1$ and $\alpha4\beta7$ integrins can mediate binding to VCAM-1, we investigated the effect of blocking antibodies to these targets administered during the challenge phase. We found using several different antibodies that either integrin could reduce the mast cell progenitor recruitment, suggesting that both these integrins contribute in the adhesion to VCAM-1. Administration of antibody to αE, the other alpha chain that pairs with $\beta7$, had no effect, indicating the specificity of the blocking. Moreover, antigen sensitized and challenged $\beta7$-integrin deficient mice also had suppressed lung mast cell progenitor recruitment compared to their wild type controls. We concluded that mast cell progenitors express functional $\alpha4\beta1$ and a$4\beta7$ integrins that interact with VCAM-1 on the lung endothelium to accomplish the transendothelial migration (Fig. 2).

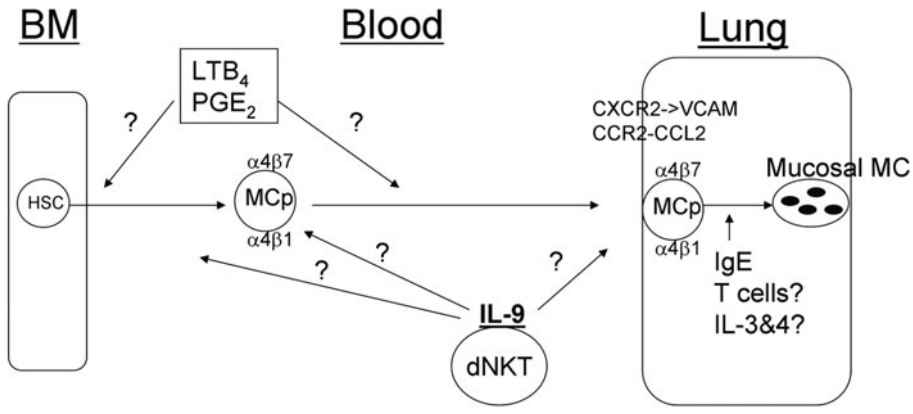

Figure 2. Identification of the molecules and cells controlling (or perhaps controlling, e.g., LTB_4, PGE_2) inflammation-induced recruitment of MCp to the lung in BALB/c mice and influencing their maturation to mature mucosal mast cells. Question marks indicate pathways not confirmed or established in vivo. See text for details.

As recruitment also requires attracting the mast cell progenitor to the site of the inflammation, we compared the antigen-induced mast cell progenitor recruitment in several different knock out mouse strains lacking the chemokine receptors CXCR2, CCR5, CCR3 and CCR2. We focused on these based on their known expression by bone marrow derived mast cells (BMMC) and/or human cord blood derived mast cells.[24,65,66] Using our established mast cell progenitor recruitment protocol, we found no diminution in mast cell progenitor recruitment in CCR5 and CCR3 knockout mice. $CXCR2^{-/-}$ mice however, had significantly reduced mast cell progenitor recruitment, 66% in total mast cell progenitor content and 53% in the concentration of mast cell progenitors per 10^6 mononuclear cells.[33] To confirm that the need for CXCR2 was due to lack of expression on the mast cell progenitor, we made bone marrow chimeras of wild type mice reconstituted with $CXCR2^{-/-}$ bone marrow. To our surprise, the $CXCR2^{-/-}$ bone marrow could reconstitute the mast cell progenitor recruitment in sublethally irradiated wild type mice, indicating that CXCR2 expression by the circulating mast cell progenitor was not needed. The opposite experiment where bone marrow chimeras of $CXCR2^{-/-}$ mice were produced by being given wild type bone marrow recapitulated the original results with the CXCR2 knockout mice indicating that a nonbone marrow-derived, relatively radioresistent host cell had to express CXCR2 for recruitment of mast cell progenitor to lung. Because CXCR2 is expressed on the lung endothelium, we hypothesized that CXCR2 expression on the lung endothelium regulates VCAM-1 expression. In accordance with this hypothesis, total mRNA levels in the lungs and protein expression of VCAM-1 on the lung endothelium was strongly reduced in CXCR2-deficient mice, suggesting that CXCR2 on the lung endothelium does regulate VCAM-1 expression and thereby the induced influx of mast cell progenitors to this tissue. In support of our observations, endothelial CXCR2 was also shown to, in part, regulate neutrophil influx in LPS-induced lung injury.[47]

Evaluation of CCR2 null mice also noted reduced recruitment in our model and consistent with this, we found that the chemokine CCL2, the major ligand for CCR2 is chemotactic for bone marrow-derived mast cell progenitors and immature (2 weeks old) BMMC cultured in SCF and IL-3. However, CCL2 was not chemotactic to BMMC cultured in IL-3 alone, even though mast cells cultured in both conditions expressed CCR2 and bound radiolabeled CCL2 with the same affinities.[67] This indicates that SCF is an important mediator in coupling CCR2 to downstream signaling events that leads to chemotaxis. When CCL2 was injected intradermally into mice, immature BMMC from IL-3 + SCF cultures administered intravenously migrated to the sites of injection. These data suggested that the CCL2/CCR2 pathway was important in recruitment of mast cell progenitors; and this was supported when we demonstrated that both CCR2- and CCL2-deficient mice had significant reductions in mast cell progenitor recruitment to the lung in our model of allergic airway inflammation. However, when we addressed whether this was due to expression of CCR2 by the mast cell progenitors by evaluating recruitment in sublethally irradiated wild type mice given $CCR2^{-/-}$ bone marrow, we found normal recruitment, showing that CCR2 expression by the circulating progenitors is dispensable. We further confirmed this by analyzing several of the mast cell colonies in the reconstituted wild type mice and found them to all be of donor ($CCR2^{-/-}$) origin. The opposite experiment, where sublethally irradiated $CCR2^{-/-}$ deficient mice are given wild type bone marrow, still had diminished recruitment, indicating a requirement for expression of the receptor by host tissues. Since mice lacking CCL2, also had diminished mast cell progenitor recruitment to lung, we also evaluated these mice using bone marrow

chimeras. We found that the critical source of CCL2 was both in bone marrow-derived cells and stroma-derived cells. Clearly, the CCL2/CCR2 axis is involved in the mechanisms that lead to antigen-induced mast cell progenitor recruitment to lung, but the process is complex and there are missing steps to be sorted out. Despite several attempts to define the critical chemokine receptor(s) and the mechanism(s) by which they act to attract the mast cell progenitor to lung in vivo, the hunt is not over.

Several other chemoattractants for mast cells may also be involved. In a study aimed at finding the chemoattractant responsible for mast cell progenitor recruitment in a similar model of OVA-induced airway inflammation, BALB/c mice were sensitized weekly with three i.p. injections with OVA in alum followed by five daily intranasal challenges and a final intranasal challenge three days later with OVA. Weller and colleagues took nasal mucosa 20 h after final OVA challenge and generated a mucosal conditional medium that was used to attract immature (2-week-old) BMMC produced in IL-3 alone.[68] Purification of the chemoattractant activity in the mucosal conditional medium demonstrated that prostaglandin E_2 (PGE_2) was inducing the chemotactic response and that an EP3 receptor antagonist could block the response. In addition, LTB_4 was shown to be chemotactically active on immature (2-week-old) BMMC.[69] Because both mediators are expressed following activation of mast cells, they are most likely to play a role in the recruitment process rather than the basal homing. Furthermore, the demonstration of an effect on 2-week-old cultured mast cells by CCL2, LTB_4 and PGE_2 with only PGE_2 attracting the more mature 10-week-old cultured mast cells, suggests a role for these factors in early recruitment, perhaps from the bone marrow rather than into the tissues.

Mast cell progenitor recruitment to allergen-challenged lung occurs in an antigen-specific manner in previously sensitized mice. Since the use of alum in the sensitization process skews the immune response towards a Th2 response, we hypothesized that Th2 cells control mast cell progenitor recruitment by providing Th2 cytokines that govern the inflammation-induced up-regulation of adhesion molecules and/or necessary production of chemoattractants. In support of this we found that the pulmonary mast cell progenitor recruitment was dependent on $CD4^+$ T cells in BALB/c mice, since depletion with monoclonal antibody before aerosol challenge inhibited the recruitment but depletion of $CD8^+$ T cells had no effect.[70] Unexpectedly, BALB/c mice lacking IL-4, IL-4R alpha chain or STAT-6 had a normal recruitment response, even though these mouse strains have diminished Th2-responses and no serum IgE.[71,72] Further, administration of blocking antibodies for the Th2 cytokines IL-3, IL-4, IL-5, IL-10 or IL-13 during the challenge phase had no effect on the recruitment. These data suggested that a Th2-response is not required for the antigen-induced recruitment of mast cell progenitors to the lung. Similarly IFN-$\gamma^{-/-}$, IL-12p40$^{-/-}$, IL12Rβ1$^{-/-}$ mice or treatment with anti-IFN-γ, anti-IL-12p40 (the IL-12 and IL-23 common chain), anti-IL-6, anti-IL-17A and anti-TGFβ1 did not reduce the recruitment, indicating no role for Th1 or for Th17 cells. Furthermore, anti-CD25 treatment had no effect on the mast cell progenitor recruitment, ruling out a critical role for T-regulatory cells. As natural killer T (NKT) cells have been implicated in the allergic pulmonary allergic response, we next evaluated a role for these cells.[73,74] Mast cell progenitor recruitment in CD1d-deficient mice, which lack all NKT cells, had significantly reduced numbers of pulmonary mast cell progenitors. Monoclonal antibody blocking of CD1d during the challenge phase also significantly reduced the mast cell progenitor recruitment compared to wild type mice treated with isotype-matched control immunoglobulins. Blockade or genetic loss

of CD1d signaling prevents the activation of both the Type 1/invariant NKT (iNKT) and the Type 2 or diverse NKT cells. However, Jα18-deficient mice that specifically lack iNKT cells can be used to differentiate between these two types of CD1d-restricted NKT cells. Surprisingly, we found that the mast cell progenitor recruitment was greater in the antigen-sensitized and challenged Jα18-deficient mice than in the wild type controls, but that anti-CD1d blocking antibody still reduced the mast cell progenitor numbers in this mutant mouse strain, indicating that it is the Type 2 NKT cells that is critical for the mast cell progenitor recruitment to the antigen challenged lung.

The screening of possible critical cytokines produced by $CD4^+$ T cells eliminated many of the mast cell-activation factors we thought might be involved. This led us to IL-9, a mast cell growth factor that is not dependent on STAT-6 for signaling.[75] Use of IL-9 deficient mice, as well as anti-IL-9 treatment with monoclonal antibodies during the challenge phase, significantly reduced mast cell progenitor recruitment. One possible source of pulmonary IL-9 is tissue resident mature mast cells. Using three different mast cell-deficient mouse strains (W^{sh}/W^{sh}, W/W^{v} and S^{l}/S^{ld}), we found that all strains had increases in mast cell progenitor recruitment comparable to their wild type controls, thereby excluding mature mast cells as the critical source of IL-9. Since NKT cells from C57BL/6 mice also had been suggested as a source of IL-9,[76] we purified iNKT cells using alpha-Galactosylceramide-CD1d tetramers, from BALB/c mice, cultured them with alpha-GalCer-pulsed bone marrow-derived dendritic cells and evaluated the supernatants for production of IL-9. These cells produced nanogram amounts of IL-9 in vitro, comparable to that produced by cultured polarized Th2 cells. To address if the requirement for IL-9 and for NKT cells were in the same pathway or parallel, we treated IL-9-deficient and wild type mice with anti-CD1d blocking antibody and CD1d-deficient and wild type mice with anti-IL-9 blocking antibody. No further reduction of the partial suppression of mast cell progenitor recruitment occurred by combining the inhibition of these two factors. These data indicates that IL-9 and the Type 2 CD1d-restricted NKT cells function in the same pathway.[70]

MAST CELL HYPERPLASIA

The increased numbers of mature mast cells associated with parasitic nematode infections and chronic allergic reactions is likely the direct result, at least in part, of the maturation of the increased numbers of mast cell progenitors called into these sites of inflammation. While current studies are exploring this area to better define the contribution of resident versus recruited mast cells, several studies have contributed to our knowledge already. In the CXCR2 null mice, the decreased influx of mast cell progenitors observed after only 3 aerosolized allergen challenges was associated with fewer numbers of interepithelial mast cells observed in the tracheal mucosa a week after the last challenge.[33] In contrast, there was no significant difference in the number of submucosal, constitutive mast cells. This finding supports a model in which the recruited mast cell progenitors give rise to the mature mucosal mast cells, but not the connective mast cell. Further work is necessary to establish the relative expansion of the connective tissue mast cells versus the relative contribution of the recruited progenitors to the increased number of mature cells.

Other factors that have been implicated in the formation of the mast cell hyperplasia include the T cell derived factors IL-3, IL-4, IL-9 and more recently IgE as a survival

and anti-apoptotic factor.[77-79] Using antibody blocking and null mice, IL-3, IL-4 and IL-9 all have been show to be required for the mucosal mast cell hyperplasia associated with a nematode infection.[77,78,80] Much less has been done though on their influence on mucosal mast cells in the lung. The critical role for IL-9 in the recruitment of the mast cell progenitor to the lung was discussed above and these studies also eliminated a role for IL-3 and IL-4 in this process in BALB/c mice. Further studies have suggested that in IL-9-deficient mice, chronic stimulation results in a small decrease in the numbers of mature mucosal mast cells, which may in turn suggest that the early dependence of recruitment is lost with further stimulation. Because of the role for IL-3 and IL-4 in the mature mast cell hyperplasia in the intestine, these bear further investigation in the lung.

Recently, a role for IgE has been found in the mast cell maturation process occurring during inflammation. Using a model of pulmonary inflammation induced by *Aspergillus fumigatus* extract that leads to remodeling and airway hyperresponsiveness, we evaluated the increase in both mast cell progenitors and the mature mast cells. Surprisingly, in BALB/c mice lacking IgE, recruitment of the immature progenitors was unaltered, but the number of mature pulmonary mast cells was significantly decreased.[79] The lack of IgE was further shown to lead to increased numbers of apoptotic cells, indicating that it acts as survival/anti-apoptotic factor.

CONCLUSION AND FUTURE PROSPECTS

During homeostatic conditions, mast cell progenitors arise in the bone marrow and are transported out to peripheral tissues via the blood. It is likely that these circulating progenitors have already made the commitment to the mast cell lineage in the adult animal, but this has not yet been confirmed. Extravasation from the blood stream occurs under basal conditions into certain tissues and is increased with inflammation. The basal homing to the intestine is highly controlled by other innate cells and by several different integrins, cellular adhesion molecules, chemokines and chemokine receptors. Many of these same components are involved in controlling the inflammation-induced recruitment to the lung, but most of the pathways are not fully understood. In addition, the inflammation-induced recruitment is dependent on other unique cells and factors that control the recruitment of these potent effectors in a tissue specific and strain specific manner. Initial studies indicate that these mast cell progenitors mature into mast cells in situ and provide the basis for the mast cell hyperplasia associated with certain inflammatory stimuli. However, this process is in need of much further work to understand the underlying mechanisms to be able to modulate this process and control the pathobiologic consequences of chronic activation of mast cells.

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CHAPTER 3

MONOMERIC IgE AND MAST CELL DEVELOPMENT, SURVIVAL AND FUNCTION

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Abstract: Mast cells play a major role in allergy and anaphylaxis, as well as a protective role in immunity against bacteria and venoms (innate immunity) and T-cell activation (acquired immunity).[1,2] It was long thought that two steps are essential to mast cell activation. The first step (sensitization) occurs when antigen-specific IgE binds to its high-affinity IgE receptor (FcεRI) expressed on the surface of mast cells. The second step occurs when antigen (Ag) or anti-IgE binds antigen-specific IgE antibodies bound to FcεRI present on the mast cell surface (this mode of stimulation hereafter referred to as IgE+Ag or IgE+anti-IgE stimulation, respectively).

Conventional wisdom has been that monomeric IgE plays only an initial, passive role in mast cell activation. However, recent findings have shown that IgE binding to its receptor FcεRI can mediate mast cell activation events even in the absence of antigen (this mode of stimulation hereafter referred to as IgE(-Ag) stimulation). Different subtypes of monomeric IgEs act via IgE(-Ag) stimulation to elicit varied effects on mast cells function, survival and differentiation. This chapter will describe the role of monomeric IgE molecules in allergic reaction, the various effects and mechanisms of action of IgE(-Ag) stimulation on mast cells and what possible developments may arise from this knowledge in the future. Since mast cells are involved in a variety of pathologic and protective responses, understanding the role that monomeric IgE plays in mast cell function, survival and differentiation will hopefully lead to better understanding and treatment of asthma and other allergic diseases, as well as improved understanding of host response to infections.

INTRODUCTION

Over the past few decades, the number of patients with allergic diseases (e.g., asthma, atopic dermatitis) has risen markedly in industrialized nations. Thirty-nine percent of

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children and 30% of adults have been diagnosed with one or more of asthma, eczema and hay fever.[3] Allergic diseases are manifested by an immune system over-reaction to a substance that the body should not normally identify as dangerous and that reaction can be something as basic as a mild irritation or it can be so extreme as to cause fatal results.

The mast cell is a type of myeloid lineage hematopoietic cell and its development and function suggest that it is involved heavily in the pathogenesis of these allergic diseases (e.g., when a mast cell is "activated" it releases chemicals into the body that cause what we call an allergic reaction). Mast cells originate from precursor cells in the bone marrow, enter the circulation and then localize to mucosal and epithelial sites under the influence of the local microenvironment.[4] At these sites, facing the outer environment and surrounding blood vessels, mast cells can produce and release a vast array of biogenic amines (e.g., histamine and serotonin), lipids (e.g., leukotrienes and prostaglandins), nucleotides (e.g., adenosine) and polypeptides (e.g., proteases, cytokines and chemokines) that act as effector molecules in allergic or inflammatory reactions as well as innate and adaptive immune responses.[5,6] Indeed, mast cells from atopic individuals that are sensitized by allergen-specific immunoglobulin E (IgE) respond to allergens by releasing mast cell-derived mediators. Subsequent allergen exposure in susceptible individuals produces a cascade of events orchestrated by immune effector cells such as Type 2 helper T (Th2) cells and eosinophils.[7] B cells stimulated by IL-4 or IL-13 produce IgE.[8]

Recent studies have shown that monomeric IgE can activate mast cells in the absence of antigen. This suggests that allergic symptoms may be due in part to the effects of monomeric IgE and that these symptoms may occur even in patients who are not exposed to allergens. Different subtypes of IgE have been shown to have different effects on mast cells. It is conceivable that different types of monomeric IgE may give rise to the range of symptoms and symptom severity observed amongst allergic patients. Anti-IgE therapy has been efficacious in treating patients with allergic diseases, pointing to the significant role IgE must have in asthma and other allergic diseases.[9] It is still unknown how anti-IgE therapy relieves allergic symptoms, but it is highly possible that preventing the effects of monomeric IgE on mast cells is a large part of how anti-IgE therapy mediates its effects. Elucidating the true role and mechanism of action of monomeric IgE's effects on mast cells will further our understanding of allergic diseases, symptoms and treatment.

This chapter will review what is known about the interaction between monomeric IgE and mast cells in the absence of antigen.

IgE+Ag STIMULATION OF MAST CELLS

The initial step in IgE+Ag mast cell activation occurs when monomeric, antigen-specific IgE binds FcεRI, stabilizing and increasing the number of FcεRI receptors on the mast cell surface.[10] This sensitizes the mast cell to a specific antigen so that the mast cell can be activated immediately upon subsequent antigen exposure. Antigen binding to antigen-specific, FcεRI-bound, IgE cross-links FcεRI (FcεRI aggregation/cross-linking), thereby inducing various mast cell activation events (e.g., degranulation, de novo synthesis and secretion of inflammatory mediators, survival promotion, migration, etc) via multiple signal transduction pathways within the mast cell.

In response to FcεRI cross-linking, various phosphorylation cascades are triggered, leading to calcium influx, nuclear importation of transcription factors, mast cell degranulation, eicosanoid generation and chemokine/cytokine production. The FcεRI

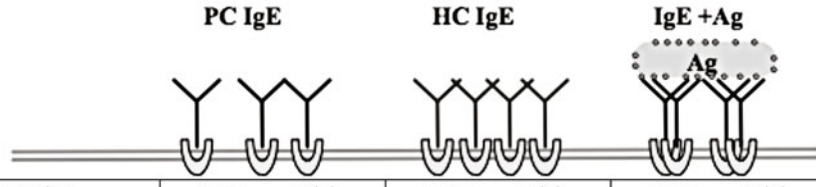
receptor consists of four subunits ($\alpha\beta\gamma_2$)—one α subunit that binds IgE, one β subunit involved in signal amplification and FcεRI stabilization and two disulfide-bonded γ subunits acting as the main signal transducer.[10] FcεRI aggregation leads to the activation of Lyn, a Src family protein tyrosine kinase (PTK) associated with the β subunit of FcεRI. The intracellular parts of FcεRI β and γ contain immunoreceptor tyrosine-based activation motifs, or ITAMs, that consist of six conserved amino acid residues spread out over an ~26 amino acid sequence, $(D/E)X_7(D/E)X_2YX_2(L/I)X_{6\text{-}8}YX_2(L/I)$.[11] Activated Lyn phosphorylates tyrosine residues in these intracellular FcεRI β and γ subunit ITAMs. β phosphorylated ITAM recruits Lyn PTK and γ phosphorylated ITAM recruits Syk PTK. Two other Src family PTKs, Fyn and Hck, also work with Lyn and Syk to activate several signaling pathways, especially the PI3K signaling pathway.[12] Signaling events activated by these PTKs (PI3K, phopholipase C/Ca^{2+} and several MAPK pathways) lead to cytokine/chemokine production and degranulation of the effector molecules mentioned in the introduction (chemicals, lipids, nucleotides and polypeptides).[13,14]

IgE(-Ag) STIMULATION OF MAST CELLS: MONOMERIC IgE AFFECTS MAST CELL FUNCTION IN THE ABSENCE OF ANTIGEN

As mentioned above, monomeric IgE appears to be involved in more than just mast cell sensitization. IgE(-Ag) induces up-regulation of FcεRI expression on the cell surface,[15-17] FcεRI stabilization,[18,19] increased histamine content,[20] histamine and leukotriene release, receptor internalization, cytokine/chemokine expression,[21] increased response to compound 48/80 and substance P,[22] F-actin content release,[23] membrane ruffling,[24] fibronectin adhesion,[25] and migration.[26] Almost all of these effects are seen with IgE+Ag and IgE+anti-IgE stimulation as well (Fig. 1). However, in IgE(-Ag) stimulation, these effects are seen at IgE concentrations 2-3 logs higher than what are required for Ag-dependent IgE+Ag mast cell activation. Since IgE(-Ag), IgE+Ag and IgE+anti-IgE can mediate similar effects at the subcellular level, it follows that IgE(-Ag) would be able to elicit mast cell responses similar to those seen with IgE+Ag and IgE+anti-IgE. Indeed, IgE(-Ag) has been shown to (i) promote mast cell survival, (ii) amplify mast cell accumulation at allergic tissue sites and (iii) mediate mast cell activation events.

(i) IgE(-Ag) stimulation seems to play a role in preventing mast cell apoptosis. Mast cells undergo apoptosis at low levels of growth factor both in vitro and in vivo. Two groups have investigated whether monomeric IgE can inhibit the mast cell apoptosis that is induced by the removal of growth factor; their results showed that increasing the concentration of monomeric IgE can indeed prevent mast cell apoptosis.[18,27] IgE(-Ag), IgE+anti-IgE and IgE+Ag (at low antigen concentrations) stimuli of FcεRI all promote mast cell survival.[28] The duration and strength of this FcεRI stimulation determines mast cell survival (further discussed under *Signal Transduction induced by Monomeric IgE*).

(ii) IgE(-Ag) and IgE+Ag are also implicated in amplification of the inflammatory response. Epicutaneous application of IgE(-Ag) and IgE+Ag at allergic tissue sites with local high IgE levels induces mast cell accumulation.[29] Mast cell accumulation can amplify inflammatory reactions via molecules such as histamine, leukotriene B_4 (LTB_4) and chemokines/cytokines released during mast cell degranulation. These effector molecules recruit other immune cells such as T cells, eosinophils, monocytes and neutrophils and induce synthesis of other pro-inflammatory effector molecules. Histamine enhances the secretion of Th2 cytokines (involved in mediating allergic responses and host defense



Concentration	Low	High	Low	High	Low	High
Receptor internal	-	-#	W	W#	W#	S
Histamine Release	-	-	W	W#	W#	S
LT release	-	-	W	W#	W#	S#
Adhesion	-	-	W#	S#	W#	S#
IL-6 production	-	-	W#	S#	W#	S#
Migration	-	-	W	S#	S#	W#
Histamine content	-	-	W	S#	W	W
DNA synthesis	-	-	W	S#	- ?	- ?
Survival	-	W#	W#	S	W#	-

Figure 1. Biological effects by various modes of stimulation via the FcεRI. Experiments using mouse BMMCs are summarized. Concentrations of stimuli are as follows: PC IgE (H1 DNP-ε-206) and HC IgE (SPE-7), 0.5 μg/ml (low) and 5 μg/ml (high); and IgE+Ag (DNP_{21}-BSA), 1 ng/ml (low) and 100 ng/ml (high). Notice that biological events are listed in a rough order of occurrences and receptor aggregation presumably occurs in all modes of stimulation, except for low PC IgE concentrations. –, not detected, -#, very weak; W, weak; W# weak~moderate; S#, moderate~strong; and S, strong. Reproduced with permission from: Kawakami T, Kitaura J. J Immunol 2005; 175:4167-4173;[28] ©2005 The American Association of Immunologists, Inc.

against parasitic infections)[30] and inhibits the production of Th1 cytokines.[31] LTB_4 recruits T cells and myeloid cells.[32-34] Transcripts coding for the CC chemokines (chemokines in which the first two cysteine residues are adjacent)[35] I-309/CCL1, MIP-1α/CCL3, MIP-1β/CCL4 and MCP-3/CCL7 are dramatically enhanced in IgE+Ag-stimulated mast cells.[36] These cytokines and chemokines recruit T cells, eosinophils, monocytes and neutrophils.

Activated mast cells may also release mediators to further induce activation of themselves and their neighboring mast cells. Adenosine and sphingosine 1-phosphate acutely released by IgE+Ag play an important role in Ca^{2+} influx, degranulation and chemotaxis to antigen.[37,38] PTK-based signaling pathways and G-protein coupled receptor (GPCR)-based signaling pathways dependent on phosphoinositide 3-kinase γ (PI3Kγ) cooperate to enhance degranulation and migration. It is likely that they induce other activation events as well. Factors that bind to GPCRs (sphingosine 1-phosphate, adenosine, LTB_4 and chemokines MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL1-3, MCP-1/CCL2 and RANTES/CCL5) contribute to IgE(-Ag) and IgE+Ag mast cell migration using both directional (chemotaxis) and nondirectional (chemokinesis) mechanisms.[26]

The ability of monomeric IgEs to attract inflammation-inducing mast cells may make it possible for amplification of inflammatory reactions to continue even after the removal of antigen as long as local IgE synthesis continues. A positive feed-back-loop hypothesis was proposed to explain inflammation seen in atopic diseases with high serum IgE levels.[14] ↑ IgE → ↑ FcεRI, ↑ mast cells → ↑ Ag-IgE and FcεRI-dependent release of IL-4, IL-13, MIP-1α/CCL3 and so on → ↑ IgE.

(iii) IgE(-Ag) stimulation exerts a vast number of effects on mast cells that all play a role in the activation of human as well as murine mast cells. For instance, IgE(-Ag) induces the production of various effector molecules. Monomeric IgE induces production

of pro-inflammatory IL-8 and monocyte chemotactic protein-1 (MCP-1) in mast cells derived from umbilical cord blood.[39] Steroids known to decrease the inflammatory response inhibit production of these chemokines. In human lung mast cells, monomeric IgE not only induces IL-8 production, but also induces mast cell degranulation, LTB_4 production and intracellular calcium influx.[40] Mast cells derived from human umbilical cord blood synthesize and release I-309/CCL1 (a ligand for CCR8 that chemoattracts Th2 cells), GM-CSF and MIP-1α/CCL3 in response to IgE(-Ag) stimulation.[41]

IgE(-Ag) also activates mast cells by increasing the number of FcεRI on the mast cell surface. Although increased numbers of FcεRI are also seen after IgE+Ag stimulation, IgE(-Ag) increases FcεRI numbers via receptor stabilization while IgE+Ag does this via actual upregulation of FcεRI expression.[42,43] Furthermore, in the case of IgE+Ag, the number of FcεRI on the mast cell surface may actually decrease at very high levels of Ag due to receptor internalization.[44]

Microarray analysis of 760 human genes showed changes in mast cell gene expression indicative of mast cell activation by IgE(-Ag) stimulation. Fifty-eight genes are upregulated after IgE(-Ag) stimulation and a total of 115 genes are upregulated during IgE+anti-IgE stimulation. The transcripts upregulated by IgE(-Ag) include many that are expected for immune cell activation: genes coding for cytokines (IL-1β, IL-6 and CSF1), chemokines (IL-8/CXCL8, MIP-1β/CCL4, MCP3/CCL7, GROα/CXCL1 and GROγ/CXCL3), cytokine and chemokine receptors, regulators of signal transduction and transcription factors, as well as other genes involved in immune regulation, cell proliferation and survival, adhesion and cytoskeletal remodeling.[45]

Finally, incubation of human cultured mast cells with IgE significantly increased the expression of two nitric oxide (NO) generating enzyme isoforms, nitric oxide synthase 2 and 3 (NOS2 and NOS3, respectively). Interestingly, IgE+anti-IgE stimulation leads to further upregulation of NOS2 expression but decreased NOS3 expression.[46] This is consistent with findings in animal asthma models and patients with mild asthma of reduced NOS3 expression 30 minutes after antigen exposure followed by a subsequent increase in NOS2 expression concomitant with increased airway reactivity.[47,48]

IgE-mediated activation phenomena have also been observed in FcεRI-expressing cells other than mast cells. For instance, monomeric IgE stimulates basophils (which are FcεRI-expressing cells) to produce CCL22 (macrophage-derived chemokine, or MDC), a chemoattractant for Th2 cells.[49] Monomeric IgE also inhibits apoptosis of neutrophils, which express FcεRI in atopic asthmatics and are thought to play a role in the development of asthma.[50] Activation of extracellular signal-related kinase (ERK), a required step for IgE(-Ag)-mediated mast cell survival, is required for differentiation of immature B cells into transitional and mature B cells.[51]

From these findings, it can be thought that in both mice and humans, monomeric IgE exerts various effects upon cells that express FcεRI, even in the absence of antigen.

MONOMERIC IgE SUBTYPES (HIGHLY CYTOKINERGIC IgE VS POORLY CYTOKINERGIC IgE)

Monomeric IgEs vary in their ability to induce different IgE(-Ag) mast cell activation events. This was brought to light by the inconsistencies found between the characteristics of monomeric IgE observed in initial studies done by Krystal et al and our research group. For instance, we found that monomeric IgE prolonged mast cell survival, but did

not affect mast cell proliferation, phosphorylation of signaling molecules, production of various cytokines and expression of apoptosis inhibitors. However, Krystal et al were able to induce almost all these mast cell activation phenomena. Another group found that monomeric IgE can induce actin polymerization.[23] It was thought that these discrepancies might have been due to a difference in the monomeric IgEs used in our experiments and various IgE molecules were examined with regards to their effect on mast cell activation and survival. As a result, it was shown that certain types of IgE molecules (highly cytokinergic IgE: HC IgE) not only prolong mast cell survival but also strongly influence mast cell degranulation and proliferation, mast cell migration[26] and adhesion to fibronection,[29] leukotriene release, production of chemokines/cytokines such as IL-6 and TNF-α, phosphorylation of signaling molecules, as well as FcεRI aggregation[21] and internalization.[44] On the other hand, there exist types of IgE molecules (poorly cytokinergic IgE: PC IgE) that do not affect mast cell activation processes other than prolongation of mast cell survival.[52] In this manner, it was shown that individual IgE molecules have different properties (Fig. 1).

There is some knowledge about the differences underlying the divergent effects of HC and PC IgE on mast cell degranulation at a molecular level. Mast cell degranulation involves fusion of the secretory granule membrane with the plasma membrane. This membrane fusion reaction is mediated by a group of integral membrane proteins called SNAREs.[53] SNAREs localize to detergent-insoluble lipid raft microdomains in FcεRI-stimulated mast cells. There are two main categories of SNAREs, vesicle SNAREs (or v-SNAREs) present on vesicle membranes in the vesicle-associated membrane protein (VAMP)/synaptobrevin family and target SNAREs (or t-SNAREs) present on target membranes in the syntaxin and SNAP-23 family. The majority of SNAP-23 is lipid raft-associated, while only small amounts of syntaxin-4 or VAMP-2 are raft-associated. One copy each of SNAP-23/syntaxin/VAMP must form a ternary complex in lipid raft membranes for membrane fusion to occur.[54] HC IgE seems to regulate VAMP-2 expression at the level of mRNA (transcriptional and/or posttranscriptional). Monomeric IgE also seems to play a role in determining the subcellular location of SNAREs. SNAP-23 and VAMP-8 protein levels were preferentially localized to lipid rafts in mast cells cultured with HC IgE and high concentrations of PC IgE when compared to mast cells cultured with low concentrations of PC IgE.[55]

Still, a clear explanation for the marked heterogeneity among IgE molecules has yet to be uncovered, as no information is truly available about the molecular differences amongst different types of IgEs. Affinity to antigen does not seem to affect IgE function—a typical HC IgE, SPE-7 and a much weaker HC IgE, H1 DNP-ε-26, have similar reported affinities (Kd = ~15 nM).[19,56] Anisotropy data has suggested that there is a difference in the extent of receptor aggregation between different IgE types. The more extensive receptor aggregation seen with HC IgE over PC IgE may help explain how a strong HC IgE can induce all activation events, whereas PC IgE can induce only a limited set of activation events to a lesser degree.[21]

The dichotomy between HC and PC IgEs is reminiscent of the variability between different human IgE molecules' ability to prime basophils for stimulation with histamine-releasing factor (HRF). HRF is a cytokine produced by macrophages and platelets. Basophils bound by IgE derived from atopic patients release histamine and cytokines such as IL-4 and IL-13 in response to HRF.[57,58] Basophils bound by IgE from normal individuals do not show this same response. IgE that has the ability to respond to HRF was termed IgE^{+} and IgE that does not have the ability to respond to HRF was

termed IgE^-. There is no information about if or how structural differences may underlie the functional differences between IgE^+ and IgE^-. However, altered usages of heavy-chain variable (V_H) genes have been reported in IgE of allergic patients. There is overrepresentation of V_H5 (one of the smallest gene families containing two members out of the total of 52 functional V_H genes) in IgE derived from allergic patients.[59] This indicates that there is indeed structural heterogeneity between human IgE molecules. Further study of human IgE heterogeneity may reveal relevance to human atopic and allergic diseases.

IgE(-Ag) STIMULATION: HYPOTHESIZED MECHANISMS OF ACTION

Questions remain about how IgE(-Ag) mediates mast cell responses similar to those seen after IgE+Ag and IgE+anti-IgE stimuli. It is established that IgE+Ag or IgE+anti-IgE requires FcεRI aggregation to mediate mast cell activation.[60] This FcεRI aggregation mechanism is consistent with the common mechanism (receptor dimerization/oligomerization) seen with receptor-mediated cell activation.[61,62] Biophysical methods, including time-resolved phosphorescence anisotropy (which can assess the size and rigidity of membrane structures by quantifying, in the nano- to microsecond range, the motion of phosphorescent dye-labeled membrane proteins that are contained within the membrane), have suggested that IgE(-Ag) stimulation also results in FcεRI aggregation.[52] Calculations have shown that IgE-IgE interactions on the cell surface (assumed to be FcεRI aggregation) took place at IgE concentrations >1 μg/mL in the absence of Ag. This is consistent with data found at several laboratories that IgE effects on survival and activation are seen at ≥ 0.5 μg/mL IgE concentrations.[28]

IgE+Ag stimulation requires localization of aggregated receptors in cholesterol/sphingolipid-rich plasma membrane domains (lipid rafts).[63,64] Lipid rafts are membrane microdomains rich in cholesterol and glycosphingolipids that have been implicated in the regulation of intracellular protein trafficking.[54] Disruption of lipid rafts prevents IgE(-Ag) induced ERK activation, suggesting that localization of aggregated receptors in lipid rafts is also necessary for IgE(-Ag) stimulation.[19]

Although these findings suggest that FcεRI aggregation is required for IgE(-Ag) stimulation, it is still unclear how IgE could cross-link FcεRI in the absence of antigen. There are several models that can be formulated regarding the mechanism of cross-linking that occurs in the absence of a specific antigen, although these proposed models do not escape the realm of conjecture. Since monovalent hapten binds to the variable (V) region and inhibits monomeric IgE effects, the V-region of monomeric IgE is likely involved somehow in mediating those IgE(-Ag) effects (such as receptor aggregation). For instance, a monomeric IgE molecule may bind to FcεRI and then use itself or another molecule to mediate FcεRI cross-linking in an Ig-Ig or Ig-X-Ig (X = unknown bridging molecule) type interaction. One type of HC IgE in particular, SPE-7 IgE, can change the initial structure of its variable region and then bind different antigens, inducing isomerization of the binding site and leading to high-affinity complexes with a deep or narrow binding site.[65] It is possible that the fragment antigen-binding (Fab) portion of FcεRI-bound IgE directly or indirectly interacts with a neighboring FcεRI-bound IgE via a third molecule that is recognized by the neighboring IgEs. Furthermore, based on findings that IPSE/alpha-1 expressed by *Schistosoma mansoni* binds to IgE,[66] it is thought that proteins derived from parasites and other organisms cross-link monomeric IgE. Studies have also shown that a 28 kDa protein, p28, may be involved in the FcεRI aggregation mediated by monomeric

IgE. p28 is associated with ~50% of FcεRI in RBL-2H3 rat mast cells in the absence of IgE, but dissociates from FcεRI at a high concentration (20 μM) of IgEs.[67] The identity of this p28 sensor protein is still unknown and further studies must be done to reveal the true involvement of p28 in IgE(-Ag) receptor aggregation. Despite the building evidence that IgE(-Ag) effects are due to binding by monomeric, not aggregate, IgE molecules, it still needs to be conclusively proven that IgE does not simply aggregate before binding FcεRI at concentrations that result in IgE(-Ag) FcεRI aggregation.[28]

The exact events that take place after IgE(-Ag)-induced FcεRI aggregation also remains to be understood. Currently, it is thought that cytokines such as IL-3 and IL-6 act in an autocrine fashion on mast cells following IgE(-Ag) stimulation. Pro-/anti-apoptotic Bcl-2 family member proteins and mitogen-activated protein kinases (MAPKs) seem to play a role in mediating the effects of IgE(-Ag). Signaling via toll-like receptors (TLRs) synergistically augment the effects of IgE(-Ag) stimulation, likely by modulating the expression of Bcl-2 family member proteins and phosphorylating MAPKs.[68]

Several findings support these proposed mechanisms. HC IgEs do induce secretion of cytokine(s) that support mast cell survival in an autocrine/paracrine manner. FcεRI aggregation appears to induce autocrine secretion of IL-3, activating IL-3R signaling pathways that promote survival.[69] Stat5 was found to be an important part of this activated IL-3R signaling pathway.[70] Neutralization of IL-3 or IL-3 deficiency drastically reduced, but did not completely prevent, HC IgE-mediated survival effects.[69] Furthermore, Krystal et al reported that monomeric IgE requires phosphorylation of ERK and intracellular calcium influx, whose signal transduction pathways result in production of reactive oxygen species (ROS) that induce IL-3 production.[71]

The cytokine IL-6 also seems involved in mast cell survival. Adding IgE to human lung mast cells (HLMC) leads to increased HLMC IL-6 mRNA expression and protein release. Anti-IL-6 antibody, but not anti-SCF antibody, prevents IgE(-Ag) mast cell survival effects. This suggests that IL-6 plays an important role in mediating IgE(-Ag) mast cell survival effects. This is consistent with the knowledge that IL-6 prevents apoptosis in HLMC and cord blood-derived mast cells (HCBMC).[72,73]

Bcl-2 family members play a key role in mediating IgE(-Ag)-induced mast cell survival. Expression of anti-apoptotic Bcl-2 family members, Bcl-xL and Bcl-2, is dependent on IL-3 and it has been reported that HC IgE prolongs mast cell survival by autocrine IL-3 production to increase the expression of Bcl-xL.[69] In line with these findings, Bcl-xL can support mast cell survival in IL-3$^{-/-}$ mast cells.[69] Also, lipopolysaccharide (LPS), a ligand for TLR4, has been shown to accentuate IgE(-Ag)-mediated mast cell survival in the absence of growth factors by working synergistically with monomeric IgE to enhance expression of Bcl-xL. LPS also works with monomeric IgE to reduce expression of pro-apoptotic Bcl-2 family members, Puma and Bim. In the absence of monomeric IgE, LPS does not have these effects.[68] It seems that multiple mast cell survival pathways converge upon the common mechanism of regulating Bcl-2 family member expression.

Phosphorylation of several MAPKs is also part of the IgE(-Ag)-induced signaling pathway leading to mast cell activation events. LPS and another TLR ligand, peptidoglycan (PGN), augment the IgE(-Ag) induction of inflammatory cytokine production by mast cells via phosphorylation of several MAPKs. Interestingly, the synergistic effects of LPS and PGN (that bind TLR4 and TLR2, respectively) are not mediated by the same set of MAPKs utilized in IgE(-Ag) signaling. The p38 MAPK inhibitor (SB203580) prevents mast cell response to HC IgE, LPS, PGN and to

combinations of HC IgE with LPS or PGN. However, while JNK inhibitor (SP600125) and ERK inhibitor (U0126) inhibit mast cell response to HC IgE, they have little effect on mast cell response to TLR ligands (LPS, PGN) and only partially inhibit response to combinations of HC IgE with LPS or PGN.[74] Thus, MAPKs are involved in IgE(-Ag)-mediated mast cell activation, but much more must be elucidated about their exact role in IgE(-Ag) activation pathways.

Interestingly, PC IgEs do not support survival of FcεRIα$^{-/-}$ mast cells cocultured with wild-type cells.[21] This indicates that PC IgEs do not induce secretion of survival-mediating cytokines in the way that HC IgEs do and that there are at least 2 different survival mechanisms evoked by IgE(-Ag) at the cellular level.[28] There may also be an IL-3-independent survival pathway used by IgE(-Ag) that requires Syk (as no survival effects of monomeric IgEs were seen in Syk$^{-/-}$ cells),[21] but the exact mechanism remains to be characterized.

IgE+Ag also seems to prevent mast cell apoptosis via an autocrine mechanism, producing IL-3, IL-4 and GM-CSF.[75] IL-4 renders mast cells reactive to the other 2 cytokines, IL-3 and GM-CSF. IgE+Ag up-regulates expression of FLIP, a caspase-8 inhibitor, to resist Fas-induced apoptosis.[76] IgE+Ag also up-regulates expression of anti-apoptotic proteins such as A1 and Bcl-xL as well as pro-apoptotic proteins such as Bim-EL and Bim-L.[77] A1 has been shown to be important for IgE+Ag-induced mast cell survival.[76] It remains to be seen if A1, Bim-EL and Bim-L play a role in IgE(-Ag)-induced mast cell survival.

SIGNAL TRANSDUCTION INDUCED BY MONOMERIC IgE

In mast cells, FcεRI aggregation induces phosphorylation of ITAMs in β and γ subunits of FcεRI, initiating specific signaling events through PTKs (Lyn, Fyn, Hck and Syk), LAT (linker for activation of T cells) and SLP-76. Activation of these signaling pathways induces mast cell degranulation and cytokine production.[78,79] At all levels of the FcεRI signaling cascade, both subtle and major similarities and differences are being revealed about how IgE(-Ag) and IgE+Ag are unique. For instance, HC IgE(-Ag) signaling induces phosphorylation of the FcεRI β subunit in a manner similar to IgE+Ag.[80] However, the phosphorylation kinetics of signaling molecules differ between the two types of stimulation.[26,27] Also, phosphorylation of unique sets of signaling molecules are differentially required for the induction of various mast cell activation events. Differences have been found between the roles of PKCβ I and II in IgE(-Ag) and IgE+Ag stimulation of bone marrow mast cells.[52,81,82] IgE(-Ag)-induced mast cell migration relies on the Lyn, Syk and Fyn PTKs and protein kinase C (PKC) β, ε and θ to a lesser degree.[26] IgE(-Ag)-mediated survival does not seem to require Fyn, Gab2, PI3K p85, or Akt.[69] Mast cell adhesion to fibronectin utilizes Lyn and Syk but not Fyn PTK, as well as other Src family kinases and PKCθ.[29]

The FcεRI γ subunit (FcRγ) ITAM is required in different ways for various mast cell functions. Also, the FcRγ-ITAM is utilized differently by IgE(-Ag) and IgE+Ag to mediate the same mast cell function.[83] IgE(-Ag) requires the α and γ subunits of FcεRI to mediate mast cell survival.[18,84] IgE(-Ag)-induced cytokine production is FcRγ-ITAM-dependent, but IgE(-Ag)-induced upregulation of FcεRI is FcRγ-ITAM-independent. IgE(-Ag)-induced receptor up-regulation also does not require Lyn, Syk, or other PTKs that are required by degranulation and other activation events.[44] The differences in FcεRI subunits and

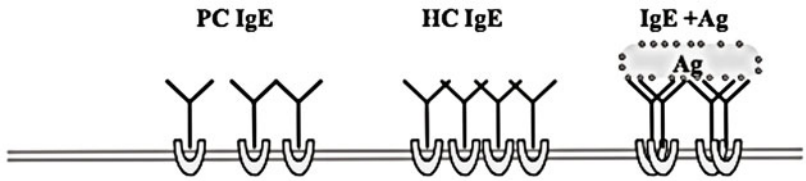
signaling molecules utilized by IgE(-Ag)-induced FcεRI up-regulation is consistent with the observation (mentioned earlier in *IgE(-Ag) Stimulation of Mast Cells (iii)*) that IgE(-Ag)-induced FcεRI up-regulation is due more to the increased lifetime of surface-resident receptors rather than to its increased synthesis and/or transport to the plasma membrane.[15,42,43]

It seems that the duration and strength of FcεRI signaling determines the type of mast cell response. Activation of ERK by activated Lyn and Syk is part of the initial FcεRI signaling pathway.[21] It is known that the duration of ERK activation alters the quality of biological responses (sustained ERK activation is required for mast cell survival but not degranulation).[85,86] The prolonged activation of ERK seen with slow on-rate IgE interaction with FcεRI is in line with the pattern of ERK activation required for survival of other cell types such as T cells.[87] The FcεRI γ subunit signaling required for survival of CD8/γ-expressing $\gamma^{-/-}$ mast cells is weak relative to the γ signaling required for degranulation.[85] These findings suggest that FcεRI signaling required for mast cell survival is prolonged but weaker than that required for mast cell degranulation.

In many receptor systems, ERK activation is regulated by Ras, which is activated by guanine nucleotide exchange factors (GEFs) such as Sos. Sos has been found to be preferentially required for FcεRI signaling by IgE(-Ag) more than IgE+Ag.[88] ERK activation in $Syk^{-/-}$ mast cells is totally abolished, showing that Syk is indispensable to IgE(-Ag)-induced mast cell survival and degranulation, as well as cytokine production.[69,71] Substrates for Syk PTK, non-T-cell activation linker (NTAL/LAB/LAT2) and LAT (both transmembrane adaptor molecules that localize to lipid rafts and possess multiple tyrosine-based activation motifs) have been shown to play a role in IgE+Ag stimulation of mast cells. These two transmembrane adaptor molecules have also been shown to play a role in IgE(-Ag)-induced mast cell activation and survival via retention of Grb2 and Sos within the plasma membrane.[88] Interestingly, NTAL/LAB/LAT2 and LAT are phosphorylated with slow kinetics upon IgE(-Ag) stimulation, but are only transiently phosphorylated upon IgE+Ag stimulation. The importance of NTAL and LAT in stabilizing Grb2-Sos within the plasma membrane is supported by the finding that Gads, a Grb2-like adaptor protein, is required for mast cell Ca^{2+} influx, degranulation and cytokine production after FcεRI aggregation.[89]

There have been quantitative differences found in the signaling pathways between IgE(-Ag) and IgE+Ag stimuli. For instance, Lyn is absolutely required for IgE(-Ag) adhesion to fibronectin and essential to FcεRI internalization. However, although Lyn is needed at low levels of antigenic stimulus, at a certain threshold and higher, antigenic stimulus can induce adhesion without relying on Lyn.[29] There are also qualitative discrepancies between the required mechanisms of action leading up to intracellular calcium influx resulting from IgE(-Ag) versus IgE+Ag stimulus.[23-25,90] HC IgE leads to a transient increase in intracellular Ca^{2+}. SK and F96365, a specific inhibitor of store-operated Ca^{2+} channels, suppresses SPE-7 IgE(-Ag) induced intracellular Ca^{2+} increase. Broad-spectrum Ca^{2+} channel inhibitors, La^{3+} or Gd^{3+}, do not. IgE+Ag induced Ca^{2+} responses are suppressed by all three inhibitors.[90] This suggests that IgE(-Ag) and IgE+Ag induce Ca^{2+} influx via different mechanisms (Fig. 2).

While IgE(-Ag) and IgE+Ag both elicit mast cell responses via FcεRI signaling pathways that utilize similar sets of signaling molecules, key differences between the signaling pathways are being uncovered. Elucidating the similarities and differences between IgE(-Ag) and IgE+Ag signaling will further our understanding of allergic disease at a cellular level.



Concentration	Low	High	Low	High	Low	High
Lyn kinase activity	-	-#	W slow	W# slow	W slow	S rapid
FceRI þtyrosine phosphorylation	-	-#	W slow	W# slow	W slow	S rapid
Syk kinase activity	-	-#	W slow	W# slow	W slow	S rapid
Increased Ca^{2+} flux	-	-#	W slow	S slow	nd	S rapid
ERK activation	-	-#	W slow	S slow	W transient	S rapid
p38 activation	-	-#	W slow	S slow	W# transient	S# rapid
Stimulation intensity:			low			high
FceRI aggregation:			weak~moderate			strong

Figure 2. Comparison of signaling events induced by different modes of FcεRI stimulation. Data with mouse BMMCs are summarized. Concentrations of stimuli are as follows: PC IgE (H1 DNP-ε-206) and HC IgE (SPE-7), 0.5 μg/ml (low) and 5 μg/ml (high); and IgE+Ag (DNP_{21}-BSA), 1 ng/ml (low) and 100 ng/ml (high). Notice that biological events are listed in a rough order of occurrences and receptor aggregation presumably occurs in all modes of stimulation, except for low PC IgE concentrations. –, not detected, -#, very weak; W, weak; W# weak~moderate; S#, moderate~strong; and S, strong. Reproduced with permission from: Kawakami T, Kitaura J. J Immunol 2005; 175:4167-4173;[28] ©2005 The American Association of Immunologists, Inc.

IN VIVO EFFECTS OF IgE(-Ag) STIMULATION

Although it is not easy to generate definitive in vivo evidence of IgE(-Ag)-mediated mast cell survival and activation, supporting data have recently accumulated. Increased mast cell numbers were seen in mice transplanted with IgE-producing hybridomas that increased serum IgE levels.[21] However, mast cell numbers were not increased in IgE-transgenic mice,[91] and the presence of IgE was not found to be a requirement for mast cell development.[92] These findings may suggest that IgE levels are not the key to maintaining homeostasis of mast cell populations in vivo. However, these findings do not undermine the previously hypothesized positive effects that IgE(-Ag) may have on mast cell survival, since the concentration of IgE must be relatively high for the survival effects of IgE(-Ag) to take place. In fact, several findings do support IgE(-Ag)-mediated mast cell survival and activation. There is in vivo evidence that IgE may be needed to maintain mast cell survival during parasitic infections. In mice infected with *Trichinella spiralis*, monomeric IgE is needed to protect the survival of mast cells.[93] In wild-type mice, *Trichinella spiralis* infection elicits an IgE response (pronounced expansion of the mast cell population in the intestine and spleen).[93] In $IgE^{-/-}$ mice, mast cells migrate from the gut to the spleen, where they undergo accelerated apoptosis compared to wildtype

mice. This suggests that high-level IgE is essential in promoting mast cell survival in vivo by preventing mast cell apoptosis in the spleen. There is also in vivo support of the proposed IL-3 autocrine mechanism for mast cell survival induced by IgE(-Ag). In IL-3$^{-/-}$ mice, mucosal mastocytosis is impaired during *Strongyloides venezuelensis* infection.[94]

Thus, in certain conditions where high levels of IgE are seen, such as parasite infection (53.3 μg/mL serum IgE in *T. spiralis*-infected mice)[93] and atopy (sometimes >100 μg/mL in NC/Nga mice),[95] monomeric IgE may play a role in regulating mast cell survival. However, it is also possible that in parasite infections, antigen is somehow involved in enhancing mast cell survival in an IgE+Ag manner, although IgE produced in response to nematode infections has not been shown to be specific to the parasite.[96]

With regards to the pathological role of IgE in vivo, Oettgen et al have studied the role of IgE in contact sensitivity using IgE-deficient mice. IgE was required for optimal sensitization but not for elicitation of contact sensitivity.[97] Mast cells were required for both sensitization and elicitation phases. Contact sensitivity was depressed in IgE-deficient mice as compared to wild-type mice, but was restored once hapten-irrelevant IgE or sensitized cells from wild-type mice were administered. During the sensitization phase, IgE was required for the generation of cytokines. Similarly, IL-6, IL-1β and MCP-1/CCL2 (and weakly TNF-α) can replace IgE in sensitization. Thus, even in the absence of a specific antigen, monomeric IgE plays a role in the contact hypersensitivity reaction. The same group also showed that inhalation of an *Aspergillus fumigatus* extract in mice induces a dramatic rise in IgE accompanied by an increase in airway activated mast cells.[98] Both mast cell expansion and activation were markedly attenuated in IgE-deficient mice. The recruitment of eosinophils to the airways was also reduced in these mice. IgE Abs were shown to sustain the survival of mature mast cells. From these findings, it can be suggested that monomeric IgE is required not only for mast cell sensitization, but also for maintenance of mast cell survival in vitro and in vivo.

MONOMERIC IgE AND MAST CELL DIFFERENTIATION

Mast cells differentiate from hematopoietic progenitor cells in the bone marrow after progenitor cells migrate to local tissues.[99-101] Stem cell factor (SCF) and its receptor, KIT, are essential for the differentiation, proliferation and survival of mast cells.[102] However, other cytokines, growth factors and other factors are also involved in the development, proliferation, survival and differentiation of mast cells.[103] Monomeric IgE acts not only upon mature mast cells, but works on mast cell progenitors to induce differentiation into mature mast cells. Bone marrow cells grown for 4 weeks in IL-3 culture supplemented with monomeric IgE, especially HC IgE, resulted in an increased number of mast cells compared with cultures grown without IgE. Also, although bone marrow cells cultured in IL-3-containing medium do eventually consist of >85% mast cells, the presence of IgE (especially HC IgE) reduces the time period required for the mast cell purity to reach ≥80%.[55]

Properties of mast cells exhibit heterogeneity, depending on what tissue and species they are derived from.[104,105] Mast cells can exhibit differences in lifespan, morphology, development, expression pattern of mouse mast cell proteases (mMCPs) and proteoglycans and sensitivity to immunologic and nonimmunologic stimuli.[106-111] Monomeric IgE binds the FcεRI receptor expressed on mast cell precursors in the early stages of mast cell differentiation to alter the expression of various genes, especially

those genes that are relevant to differentiation. As a result, different IgE subtypes can modulate the mast cell phenotype in various ways and likely play a role in giving rise to mast cell heterogeneity.

For example, the expression of early growth response factor-1 (Egr-1) is upregulated in cultures containing high and low concentrations of HC IgE and a high concentration of PC IgE. Egr-1 is a transcription factor involved in the production of TNF-α in mast cells. TNF-α helps mediate IL-3-dependent differentiation of mast cells.[112] TNF-α expression was increased in bone marrow cells cultured with HC IgE and high concentrations of PC IgE (consistent with the increased Egr-1 expression seen with HC IgE and high concentrations of PC IgE). However, levels of TNF-α protein were barely detectable even in bone marrow cells cultured with HC IgE. Expression of IL-13 and TGF-β is comparable in bone marrow cells cultured with different subtypes of IgE.[55]

These findings suggest that monomeric IgE induces mast cell differentiation and plays a role in determining mast cell phenotype.

POLYCLONAL IgE AND MAST CELL ACTIVATION

Although there is evidence that monoclonal IgE acts in an IgE(-Ag) manner to induce mast cell activation and mast cell differentiation, the effect of polyclonal IgE on mast cells has not been studied until recently. A mixture of equal amounts of several different types of monoclonal monomeric IgE promoted cell survival and induced cytokine production at the level that would be expected based on the average of the different types of monomeric IgE.[21] This suggested that polyclonal IgE would also be able to affect mast cell survival and function.

Polyclonal monomeric IgE taken from the serum of mice with atopic dermatitis induced by epicutaneous administration with mite antigen and Staphylococcus Enterotoxin B (SEB)[113,114] resulted in prolonged mast cell survival and cytokine production in comparison to serum from mice without atopic dermatitis. This prolonged mast cell survival and cytokine production was not seen when IgE was removed from the blood or when the serum utilized was from IgE-deficient mice with atopic dermatitis. Also, prolonged mast cell survival and cytokine production was not seen when polyclonal monomeric IgE from the serum of mice with atopic dermatitis was added to mast cells from FcεRIα-deficient mice. These results suggest that polyclonal IgEs mediate their effects via FcεRI in a similar manner to monoclonal IgEs. The effect of human polyclonal monomeric IgE, from the serum of patients with atopic dermatitis, on human peripheral blood-derived cultured mast cells was additionally studied. IL-8 production was increased in mast cells cultured with serum taken from patients with atopic dermatitis when compared to mast cells cultured with serum added from healthy subjects. Removing IgE from the serum inhibited IL-8 production.[115]

Previous studies have dealt with the effects of monoclonal monomeric IgE. However, monomeric IgE in the serum of actual patients is polyclonal. The results of these polyclonal monomeric IgE studies are of great significance, because they provide the first evidence that polyclonal monomeric IgE in the serum of patients does actively stimulate mast cells. Better understanding the effects of polyclonal monomeric IgE is the next step in proving that the effects of monomeric IgE play an active role in the development of allergic diseases.

CONCLUSION

Mast cells play a major role in allergy, anaphylaxis, innate immunity and acquired immunity. The role of monomeric IgE was previously thought to be limited to passive sensitization of the mast cell for IgE+Ag activation. It is now understood that monomeric IgE plays a much larger role in mast cell function and can induce mast cell activation events via IgE(-Ag) stimulation. The mechanisms of action by which IgE mediates its effects on mast cells via IgE+Ag and IgE(-Ag) stimulation are being uncovered. FcεRI aggregation seems to play a large role, as do the signaling molecules Lyn, Fyn, Hck and Syk. Evidence supports the role of autocrine IL-3 secretion in IgE(-Ag) stimulation. Experiments being done studying the differences between HC IgE and PC IgE suggest that different subtypes of monomeric IgEs elicit different sets of mast cell responses and that the different IgE subtypes may work via different mechanisms of action. While there is a lack of definitive evidence of IgE(-Ag) effects in vivo, there have been promising studies, especially those looking at mast cell survival in mice with parasitic infections. The more recent studies have elucidated a role for IgE(-Ag) in mast cell differentiation in addition to its previously acknowledged role in mature mast cell survival and activation. The most recent studies have also found that polyclonal monomeric IgE has effects on mast cell function similar to those seen with monoclonal monomeric IgE stimulation.

The clinical importance of these findings lies in the fact that monomeric IgE plays a larger role in mast cell function, survival and differentiation than previously believed. Since the mast cell is involved in allergy, anaphylaxis and both innate and acquired immunity, better understanding of the effects and mechanisms of action of IgE(-Ag) stimulation will lead to an improved understanding of these pathologic and protective responses. This, in turn, may lead to the uncovering of better treatments for asthma and other allergic diseases, as well as ways to improve host response to infections.

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CHAPTER 4

MAST CELL APOPTOSIS AND SURVIVAL

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Abstract: Mast cells are enigmatic cells that are recognized as critical components of our immune system. They are strategically localized at the host/environment interface, display a long lifespan once situated in tissues and have the ability to produce, store and upon activation, release immuno-regulatory molecules. In specific acute and chronic conditions, mast cell accumulation, activation and release of mediators are important for the initiation and perpetuation of the inflammation associated with these disease states. During the past decade, regulatory pathways for mast cell survival have been elucidated, which have, in part, helped to explain the increased number and survival of mast cells observed during inflammatory reactions associated with, e.g., the allergic response. One key group of regulators involved in cell survival and apoptosis is the Bcl-2 family of proteins. The Bcl-2 family consists of pro- and anti-apoptotic members, where the balance between these members determines cellular fate via protein-protein interactions. In this chapter, we will discuss the regulation of mast cell apoptosis and survival and how understanding the mechanisms by which Bcl-2 family members regulate mast cell survival could lead to the identification of key proteins that affect the severity of inflammation. This knowledge could be used to develop treatments for mast cell disorders such as mastocytosis and other inflammatory diseases where mast cells are involved.

INTRODUCTION

Mast cells have primarily been regarded as effector cells in allergic disorders, releasing pro-inflammatory mediators upon antigen/IgE-dependent FcεRI crosslinking. However, their strategic distribution and their capacity to secrete potent factors, indicate that they can take part in various immune responses. There has been a recent renaissance in mast cell research which has resulted in an appreciation of their other

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roles beyond atopic diseases.[1] In this respect mast cells are now recognized to play a key role in both host defence and in many different diseases, where they either can be protective or promote the disease.

An accumulation of mast cells is often observed at sites of both bacterial and viral infections, or in parasitic diseases where the release of mast cell mediators contributes to an immune response (reviewed in ref. 2). During viral infections, mast cells release mediators distinct from those induced by a bacterial infection and these mediators then selectively recruit T cells and natural killer cells, rather than neutrophils, to the site of infection.[3] In response to nematodes and other parasites, mast cells and in particular mast cell protease (MCP)-1[4] is critical for parasite expulsion. Mast cells have also been associated with autoimmune diseases, such as multiple sclerosis (MS) and rheumatoid arthritis (RA). The most compelling evidence for the involvement of mast cells in MS comes from the use of an animal model of experimental allergic encephalomyelitis (EAE) where mice lacking mast cells develop less severe EAE and at a later onset.[5] In RA, mast cells have been suggested to contribute to the chronic inflammation and destruction of joints.[6] However, the recent finding that mast cell-derived IL-10 could limit leukocyte infiltration, inflammation and tissue damage in a mouse model of contact dermatitis[7] cautions us not to see mast cells as simply detrimental for a disease.

Mast cell numbers have been found to increase within the airway smooth muscle of asthmatic patients[8] and the current view is that mast cells contribute to the immediate reaction and to the progression of the later chronic reaction (reviewed in ref. 9). Mast cells are also often found in association with many different tumours; and studies indicate that mast cells are associated with angiogenesis in some tumours and can promote growth and metastasis via the release of various mediators.[10-12] These findings imply that mast cells might participate in tumorogenesis rather than providing a defence against these tumours.

Since mast cells appear to be involved in a variety of diseases, the possibility to use them as direct targets for new therapeutical interventions is attractive. A potential way of doing this is through the regulation of mast cell numbers and their activity within tissues, thereby affecting the initiation, perpetuation and thus the severity of inflammation.

MAST CELL BIOLOGY

Mast cells are found in tissues throughout the body but originate from haematopoietic stem cells in the bone marrow.[13,14] Unlike many other haematopoietic cells that leave the bone marrow fully matured, mast cells stem from immature precursors that leave the bone marrow to circulate in the blood. Once the cells are recruited into the peripheral tissues they can, under the influence of stem cell factor (SCF) and other locally produced cytokines, differentiate into mature mast cells.[15] The surrounding microenvironment in tissues affect mast cell numbers, as well as their protease content and effector profile, so that their phenotype reflects the local milieu they are situated in.[16]

Mature mast cells do not represent a homogenous population and are usually divided into two main subtypes. Rodent mast cells are classified into connective tissue mast cells (CTMC) and mucosal mast cells (MMC).[17] MMCs depend, in addition to SCF, on T-cell-derived cytokines, whereas CTMCs primarily require SCF for their persistence. Mice deficient in T cells lack the MMC subset, whereas the CTMC subset has no T-cell dependence and subsequently appear at normal numbers.[18] Human mast cells are classified,

based on the expression of proteases in their granules, into mast cells containing tryptase (MC_T) and mast cells containing tryptase and chymase (MC_{TC}).[19] In terms of tissue localization, human MC_T correspond to rodent MMC, being located predominantly in mucosal tissues, whereas human MC_{TC} correspond to rodent CTMC cells and are mainly found in connective tissues.[20] This is also consistent with the finding that humans with a T-cell immunodeficiency lack MC_T in the intraepithelial compartment of their intestine, while MC_{TC} appear at normal numbers in the surrounding connective tissues.[21]

Mast cells normally survive within tissues for several months and their numbers are kept relatively constant. However, intestinal helmith infection of rodents will generate a T_H2-type inflammatory response, resulting in MMC hyperplasia which is needed for the expulsion of the worm.[22] Mast cells arising during the T_H2 immune response undergo dramatic changes in their granule architecture; and their content of proteases and proteoglycans, due to the milieu of T-cell-derived cytokines.[22-24] Similarly, the influence of T_H2-derived cytokines can be seen in allergic diseases of humans, where they are thought to contribute to the alterations of phenotype, effector profile and increase of mast cell numbers in the mucosal epithelium of the nose, bronchi and gastrointestinal tracts,[25] linking T_H2-type inflammatory responses with mast cell hyperplasia.[26]

At sites of tissue inflammation, mast cells accumulate and orchestrate the inflammatory response. Mast cell numbers increase in several inflammatory conditions such as allergy, asthma, various skin diseases, autoimmune disease like rheumatoid arthritis and tumours. In several of these diseases, a correlation between mast cell numbers and disease severity has been described.[27-31] Therefore, it would be desirable to be able to regulate mast cell numbers and their activity within the tissue, with the hypothesis that a reduction of mast cell numbers would generate lesser symptoms.

REGULATION OF APOPTOSIS AND SURVIVAL

Apoptosis, or programmed cell death, is vital for the development and health of a multicellular organism. Apoptosis is used during the development of an organism to sculpture organs and tissues and to ensure immunity. Later in life, apoptosis protects by executing deletion of damaged, aged or potentially dangerous cells. Thereby homoeostasis, the balance between cell proliferation and death, is maintained.

During apoptosis, cells die in a controlled, regulated way in response to various stimuli such as growth factor deprivation, DNA damage, developmental signals, cellular stress and cytotoxic drugs. The sensitivity of cells to apoptotic signals can vary depending on a number of factors such as the nature of the stimuli, the stage of the cell cycle and the expression of pro- and anti-apoptotic proteins. Dysregulation of apoptosis can lead to either an excess or a deficit in cell death and is associated with a variety of diseases.[32] In cancer and autoimmune diseases, cells commonly escape apoptosis by overexpressing anti-apoptotic proteins[33,34] thus making inhibition of these proteins an attractive strategy for therapy.

Apoptosis is regulated via two parallel but converging pathways, the extrinsic and the intrinsic pathway (Fig. 1). Both pathways commonly involve caspase activation and lead to targeted degradation of cellular structures and formation of apoptotic bodies.[35] The extrinsic pathway is stimulated via external signals from the environment outside the cell.[36] The signal is transmitted through death receptors, belonging to the TNF receptor family (such as Fas/CD95R and the receptor for Tumor necrosis (TNF)-related

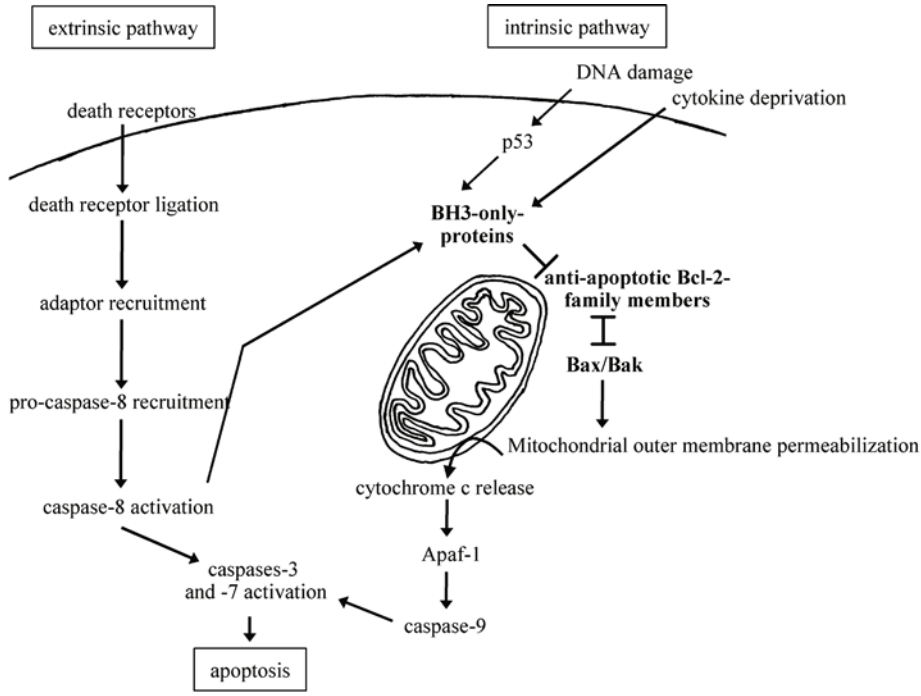

Figure 1. The extrinsic and intrinsic pathway of apoptosis. Left: The extrinsic pathway-induced apoptosis is triggered by ligation of death receptors (such as Fas or TRAIL). Death receptor stimulation-mediated signalling can activate caspase-8 directly, which leads to caspase-3 activation and finally cell death. Upon death receptor stimulation activated caspase-8 cleaves Bid into truncated Bid (tBid) that can interact with the intrinsic pathway. Right: The intrinsic pathway responds to stress factors such as DNA damage, UV radiation and cytokine deprivation that initiate apoptosis by activating the BH3-only proteins. These can neutralize the anti-apoptotic proteins which lead to activation of Bax and Bak. Some of the BH3-only proteins may also be able to directly activate Bax and Bak. Once activated, Bax and Bak cause outer mitochondrial disruption and release of cytochrome c that together with caspase-9, Apaf-1and ATP form an apoptosome that trigger the activation of caspase-9 and downstream effector caspases like caspase-3 that execute cell death.

apoptosis-inducing ligand (TRAIL-R), located on the surface of the cell; and which lead to the activation of the caspase cascade via caspase-8 (caspase-10 in humans). The intrinsic or mitochondrial pathway responds to stress factors such as growth factor deprivation, DNA damage and other stimuli that originate from the inside of the cell.[37,38] The Bcl-2 family of proteins plays a key role in the intrinsic pathway of apoptosis. Their primary site of action is at the mitochondrial membrane where the relative balance of Bcl-2 family members determines if mitochondrial outer membrane permeabilisation (MOMP) will occur. Following MOMP, apoptogenic factors, such as cytochrome c, will be released. This leads to the activation of Apoptotic protease activating factor 1 (Apaf-1) and the formation of the apoptosome; consisting of cytochrome c, Apaf-1 and adenosine triphosphate (ATP). The apoptosome activates caspase-9 which in turn induces the activation of effector caspases that cause proteolytic degradation and subsequent cell death.[39]

Since caspases are effectors of both the extrinsic and intrinsic pathways, their activities need to be strictly regulated. The Inhibitor of Apoptosis Proteins (IAP) family interacts with caspases and as a consequence inhibits their proteolytic activity.[40] IAP members in turn are regulated by the Smac/DIABLO protein which is released from mitochondria during the apoptotic process.[41] Although the two pathways are independent from each other[42] crosstalk does occur. This is mediated via Bid, a member of the Bcl-2 family, which can be activated by caspase-8 (or caspase-10 in humans)-mediated proteolysis which generates two fragments. The truncated Bid fragment (tBid) has the capacity to influence the intrinsic pathway.[43]

The Bcl-2 protein family contains members that safeguard cell survival (anti-apoptotic proteins) as well as proteins that promote apoptosis (pro-apoptotic proteins). So far, more than a dozen members have been identified. The Bcl-2 family members are characterized by the presence of at least one and up to four Bcl-2 homology (BH) domains. The anti-apoptotic members (including Bcl-2, Bcl-XL, Bcl-w, A1/Bfl-1 and Mcl-1) contain up to four BH domains (BH1-4) and a trans-membrane region (TM) that allows targeting to intracellular membranes. The pro-apoptotic members can be sub-divided into two groups: BH3 domain only proteins (BH3-only; such as Bcl-XS, Bad, Bim, Bmf, Bid, Puma and Noxa), which share with each other only the BH3 domain; and Bax/Bak-like proteins (Bax, Bak and Bok; a non-extensively studied Bax/Bak-like protein), which contain BH1, BH2 and BH3 domains and a trans-membrane region.[39,44]

Regulation of apoptosis is dependent on the balance between the different Bcl-2 family members and through the interactions between the proteins within the three subgroups. Individual BH3-only proteins differ in their ability to bind to anti-apoptotic Bcl-2 family members and this specificity governs the function of these molecules[45,46] (Fig. 2). The Bim, Puma and tBid proteins, being more promiscuous in their binding

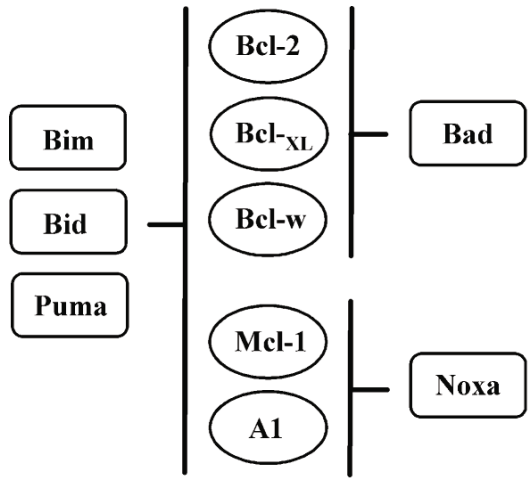

Figure 2. BH3-only proteins differ in their ability to bind to anti-apoptotic Bcl-2 family members. Bim, Bid and Puma binds promiscuously to all anti-apoptotic proteins while Bad only binds to Bcl-2, Bcl-XL and Bcl-w and Noxa to A1 and Mcl-1.

to anti-apoptotic members, have the capacity to induce apoptosis on their own, while the more selective Noxa and Bad must cooperate in order to do this. Following the interaction of BH3-only proteins and anti-apoptotic Bcl-2 proteins, which targets the cell for apoptosis, execution requires activation of Bax/Bak-like proteins.[47,48] Although it is well-known that Bcl-2 family members do interact, different modes of action have been proposed for the interactions between anti-apoptotic and pro-apoptotic Bcl-2 family proteins (reviewed in ref. 29).

Targeting the Bcl-2 family is one of the strategies for the development of therapeutic treatments against diseases associated with dysregulation or dysfunction of apoptosis. A way of targeting the Bcl-2 family has been the development of drugs that mimic the binding of BH3-only proteins to the anti-apoptotic proteins, thus inhibiting their function and promoting apoptosis. This has already proven useful in cancer therapy where the use of BH3 mimetics sensitizes tumour cells to apoptosis.[50]

MAST CELL APOPTOSIS

Mature mast cells situated in tissues are long-lived and primarily depend on SCF for their survival. The importance of SCF is highlighted by the almost complete lack of mast cells in mice deficient in Kit or SCF[51-53] and reducing SCF production reduces mast cell numbers in tissues.[54] In vitro-derived mast cells deprived of their requisite growth factors and thereby destined for apoptosis can be rescued by the addition of SCF.[55,56] Furthermore, inhibition of Kit using the specific tyrosine kinase inhibitor imatinib induces mast cell apoptosis in the synovia.[57] It has been shown that SCF regulates mast cell survival through inactivation of FOXO3a and down-regulation and phosphorylation of its target, the pro-apoptotic BH3-only protein, Bim[58] subsequently leading to the ubiquitination and proteasomal degradation of Bim.[59-61] Moreover, Bim-deficient mast cells are also found to be partially resistant to cytokine deprivation-induced apoptosis in culture.[62] Since overexpression of Bcl-2 protected mast cells more potently than loss of Bim, other pro-apoptotic BH3-only proteins, besides Bim, are likely to be involved in this process.

A way to unravel the role of Bcl-2 family members in the regulation of mast cell survival and apoptosis is the use of Bcl-2 family gene-deficient mice and cells. The role of other BH3-only proteins, besides Bim, in the regulation of mast cell survival and apoptosis, has recently been elucidated. This has lead to the identification of the BH3-only protein Puma as critical for the induction of mast cell apoptosis following cytokine deprivation. Upon cytokine deprivation-induced apoptosis, both Bim and Puma are upregulated with resulting induction of apoptosis in the mast cells[58,63] and mast cells lacking either Bim or Puma show an increased viability upon cytokine deprivation compared to wild type (wt) mast cells. The combined loss of both Bim and Puma offers the same level of protection as overexpression of Bcl-2, demonstrating the importance of a tight regulation of Bim and Puma for mast cell survival, since both bind with high affinity to all anti-apoptotic Bcl-2 family, members inhibiting their effects.

The importance of the effector proteins Bax and Bak is demonstrated by the fact that their absence abolishes most apoptotic responses sensed by BH3-only proteins.[47] Loss of both Bak and Bax results in embryonic lethality,[64] whereas mice deficient in Bax or Bak will develop more or less normally.[64,65] Although both Bax and Bak

have been shown to have largely overlapping functions in many cells, it has also been reported that one of them can have a dominant role over the other in certain cell types.[64,66] Increased mast cell numbers are found in the stomach mucosa of mice lacking Bax or Bak; and in the back skin of mice lacking Bak.[67] This may indicate a more prominent role for Bax in MMCs compared to CTMCs for cell homeostasis. Furthermore, loss of Bax conferred protection from cytokine-deprivation induced apoptosis of in vitro-derived BMMCs.[67] A recent study demonstrates both Bax and Bak are present in in vitro-derived mast cells, although the levels of Bax appear to exceed that of Bak.[68] Upon cytokine withdrawal, Bak- deficient mast cells died at a similar rate as wt mast cells, whereas Bax-deficient mast cells and mast cells deficient for both Bak and Bax were partially or completely resistant to apoptosis, respectively. Notably, only combined loss of both *bax* and *bak* confers complete resistance to cytokine deprivation-induced apoptosis, which demonstrates that activated Bak does play a role in mast cell apoptosis, though to a lesser extent than activated Bax. The total resistance seen in mast cells deficient for both Bak and Bax was comparable to mast cells deficient in both pro-apoptotic Bim and Puma, or mast cells overexpressing anti-apoptotic Bcl-2. Downstream of the Bax and Bak proteins, Apaf-1 and caspase-9 carry out their functions in the intrinsic pathway of apoptosis. It has been shown that lymphocytes lacking either of these proteins still have the capacity to undergo apoptosis in response to growth factor deprivation or DNA damage.[69] In contrast, mast cells deficient for both Apaf-1 and caspase-9 fail to undergo apoptosis in response to cytokine deprivation and instead remain in a 'senescent' stage with no proliferative capability and with lost effector functions.[70]

Many Bcl-2 family member gene deletions in mice lead to disrupted homeostasis and, as a consequence, cells accumulate in excess. This might cause increased tumour incidence and autoimmunity in mice reaching adulthood.[71,72] The lack of certain Bcl-2 family members can have implications during the development of mast cells both in vitro and in vivo. Mice deficient in anti-apoptotic A1 will develop a normal phenotype and display mast cell numbers in the tissues comparable to wild-type mice.[73,74] In contrast, anti-apoptotic Bcl-2, Bcl-XL, Mcl-1, or Bcl-w deficiency is detrimental during development in mice.[75,76] In the absence of the genes *bcl-x* or *bcl-2*, no offspring are born or are born with a short life expectancy, respectively.[76,77] It is, however, possible to generate mast cells from murine embryonic stem cells lacking *bcl-x* or *bcl-2*, although this requires both SCF and IL-3 and produces significantly fewer mast cells compared to wild type embryonic stem cells.[78]

The importance of the anti-apoptotic Bcl-2 protein for mast cell survival is also highlighted by the fact that inhibition of Bcl-2, using single chained antibodies against Bcl-2, induces mast cell apoptosis. The protein-protein interaction of Bcl-2 is then specifically neutralized intracellularly, thereby inhibiting its anti-apoptotic function.[79]

Since mast cells can undergo apoptosis via the intrinsic pathway, it seems likely that mast cells might also undergo apoptosis in response to activation through death receptors/the extrinsic pathway, thus providing an additional pathway that could contribute to the regulation of mast cell numbers. The expression of death receptors Fas/CD95R and TRAIL-R have been identified on both murine and human mast cells, although induction of apoptosis through these receptors varies. Fas/CD95R activation has been found to induce apoptosis in murine mast cells,[80] whereas TRAIL-R crosslinking causes an increase in human mast cell apoptosis.[81]

MAST CELL SURVIVAL

Mast cells have the ability to survive the aggregation of FcεRI (IgE/FcεRI crosslinking) and the subsequent degranulation process which is a key event in perpetuating the inflammatory response.[82,83] This increased survival of both human and murine mast cells (activation-induced survival) following IgE/FcεRI crosslinking has been demonstrated.[74,84-86] A fundamental question in mast cell biology is how this survival is mediated. Opposing results have however, been reported regarding the effects of enhanced mast cell survival following IgE/FcεRI crosslinking.[87,88] These differences may be due to IgE type, strength and duration of the crosslinking, or the mast cell phenotype.[85,89-91] One study showed that weak to moderate FcεRI crosslinking led to activation-induced survival, while stronger FcεRI crosslinking resulted in increased degranulation and IL-6 production but diminished survival.[85] Moreover, with the use of a CD8 fusion protein, the strength of FcεRI signals was manipulated, demonstrating activation-induced survival even in the absence of degranulation.[90]

Beside the nature of the stimuli, in vivo findings suggest the subtype influences the mast cell response. There is a local accumulation of IgE bearing MC_Ts upon Ag exposure in seasonal allergic rhinitis, whereas MC_{TC}s do not increase.[92] In vitro, mast cell populations have been shown to differ in their ability to survive allergic reactions, where the phenotype of the mast cells seems to determine if the cells are to induce a survival program upon allergic activation or not. While cytokine-deprived mast cells of connective tissue-like subtype exhibit FcεRI stimulation-induced upregulation of anti-apoptotic A1 and enhanced cell survival in vitro, mast cells of the mucosal-like type do not.[91]

Upon FcεRI crosslinking, mouse bone marrow derived mast cells (BMMCs) upregulate anti-apoptotic Bcl-2 family member A1[74] and Bcl-XL and also, to a lesser extent, Bcl-2 on an mRNA level.[62,93] It has also been shown that mouse mast cells deficient in A1 do not exhibit activation-induced survival upon FcεRI crosslinking.[74] Interestingly, A1-deficient mice display mast cell numbers in the tissue comparable to wild type (wt) mice.[74] However, upon sensitization and subsequent provocation with allergen, A1-deficient mice show a lower number of mast cell compared to wt mice.[74] The human homologue of A1, *bfl-1*, is upregulated in human mast cells upon FcεRI aggregation[94] and it was also shown that human cord blood derived mast cells (CBMCs), following FcεRI crosslinking, upregulate the anti-apoptotic Bcl-2 family member Mcl-1.[88,94] These observations provide an explanation for IgE-mediated activation-induced mast cell survival, demonstrating a major role of A1 in murine mast cells and suggest that the human homologue Bfl-1 could be of importance in human mast cells for this process.

Interestingly, FcεRI crosslinking not only regulates anti-apoptotic proteins of the Bcl-2 family, but also pro-apoptotic proteins are affected. In murine mast cells, FcεRI crosslinking upregulates and induces a rapid, but transient, phosphorylation of Bim.[62] Other BH3-only proteins including Bad are also phosphorylated, thereby inhibiting their pro-apoptotic functions.[95] In CBMCs, FcεRI crosslinking increases TRAIL-induced caspase-8 and caspase-3 activation; and the levels of FLICE-like inhibitory protein (FLIP), Mcl-1 and Bim are upregulated while Bid is downregulated.[88] The conundrum of Bim upregulation following FcεRI crosslinking needs further investigation. It has been described that the interaction between A1 and Bim increases the half life of A1 and amplifies its anti-apoptotic effect[96] and it is possible that the transient phosphorylation of Bim inhibits its apoptotic effect.[62]

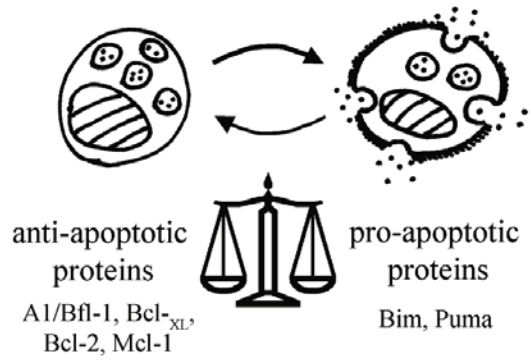

Figure 3. The balance and interactions between different anti-apoptotic and pro-apoptotic activities/ proteins determine whether the mast cell will survive or undergo apoptosis upon IgE/FcεRI crosslinking.

Given that pro- and anti-apoptotic Bcl-2 family proteins can bind to each other and titrate each others function suggests that the regulation of the aforementioned Bcl-2 family members, both anti-apoptotic as well as pro-apoptotic, following FcεRI crosslinking is likely to determine cell fate (Fig. 3).

MASTOCYTOSIS

Mastocytosis is a disorder characterized by an abnormal accumulation of malignant mast cells in one or several organs.[97] In systemic mastocytosis, most of the neoplastic mast cells have an activating mutation in *c-kit,* the receptor for SCF, causing an auto-activation of the receptor.[97-100] In mast cells, SCF normally regulates survival by repressing the levels of pro-apoptotic BH3-only protein Bim.[58] SCF affects both the transcription of Bim by inactivating its transcription factor and by phosphorylation which leads to proteasomal degradation of the protein. Recent data suggests Bim is expressed at low levels in neoplastic mast cells in systemic mastocytosis; and that drug-induced re-expression of Bim is associated with inhibition of proliferation and diminished survival.[101]

Furthermore, neoplastic mast cells express the anti-apoptotic Bcl-2 family protein Mcl-1 constitutively; and targeting Mcl-1 in these cells is associated with reduced growth and induction of apoptosis.[102] This also suggests the anti-apoptotic Bcl-2 family member Mcl-1 is a novel, interesting target in treating mastocytosis. An overexpression and dysregulation of pro-survival Bcl-2 and Bcl-XL has also been detected in mastocytosis and may also contribute to the pathogenesis of the disease.[103-105] Thus, small molecular inhibitors of crucial Bcl-2 family members involved in mast cell survival and/or apoptosis offer an attractive strategy for the treatment of this disease.

CONCLUSION

Mast cells have a central role in the initiation and perpetuation of inflammatory responses associated with allergy and asthma. Reducing the severity of this inflammation

by decreasing mast cell numbers, for example through induction of apoptosis, has been suggested as an alternative therapeutic treatment in mast cell-associated diseases. In order to achieve this objective, we need to understand how mast cell survival and apoptosis is regulated in these settings. Pro- and anti-apoptotic proteins belonging to the Bcl-2 family are known to regulate apoptosis and, although our knowledge of how these regulate mast cell survival and apoptosis is advancing, the structural and functional differences between the members and their relative impact on mast cell survival and apoptosis need to be clarified. It is known, however, that anti-apoptotic Bcl-2 family proteins bind to and neutralize, pro-apoptotic members. This suggests that, once the key Bcl-2 family members that affect the severity of inflammation have been identified, the use of small molecular inhibitors could block their binding and thereby sensitize mast cells for apoptosis.

A way of targeting the Bcl-2 family has been the development of drugs that mimic the binding of BH3-only proteins to the anti-apoptotic proteins thereby inhibiting their function and thus promoting apoptosis. This has already proven useful in cancer therapy where the use of BH3 mimetics sensitizes tumour cells to apoptosis.[50] ABT-737 is a small molecular inhibitor, mimicking the binding BH3 domain of the pro-apoptotic protein Bad. It binds with high affinity to the anti-apoptotic proteins Bcl-XL, Bcl-2 and Bcl-w but not Mcl-1 or A1.[106] It has been demonstrated to be efficacious in the treatment of solid tumors and lymphoid malignancies, although overexpression of Mcl-1 can make cells refractory to the drug.[107-111]

In allergy, a way to sensitise mast cells for apoptosis would be to inhibit the interactions of pro-survival A1/Bfl-1 with its, as yet, unidentified binding partner.

In allergy a way to sensitise mast cells for apoptosis would be to inhibit the interactions of pro-survival A1/Bfl-1 with its yet not identified binding partner by small molecule inhibitors. Many studies on the anti-apoptotic function of A1/Bfl-1 and its interactions with other Bcl-2 family members have been performed in systems where A1/Bfl-1 was overexpressed. Hopefully the recent derived crystal structures of both A1[112] and Bfl-1[113] will add to our understanding of how A1/Bfl-1 exert its anti-apoptotic function and aid when designing a small molecular inhibitor to abrogate the interactions of anti-apoptotic A1/Bfl-1 with its yet unidentified binding partner.

Although systemic abolishment of mast cells is not desirable, targeting of mast cells within a specific tissue could have beneficial therapeutic effects. New strategies targeting mast cell survival should decrease the severity of inflammation in mast cell-associated disorders.

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SECTION III

MAST CELL ACTIVATION

CHAPTER 5

REGULATORS OF Ca^{2+} SIGNALING IN MAST CELLS:

Potential Targets for Treatment of Mast Cell-Related Diseases?

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Abstract: A calcium signal is essential for degranulation, generation of eicosanoids and optimal production of cytokines in mast cells in response to antigen and other stimulants. The signal is initiated by phospholipase C-mediated production of inositol 1,4,5-trisphosphate resulting in release of stored Ca^{2+} from the endoplasmic reticulum (ER) and Golgi. Depletion of these stores activates influx of extracellular Ca^{2+}, usually referred to as store-operated calcium entry (SOCE), through the interaction of the Ca^{2+}-sensor, stromal interacting molecule-1 (STIM1), in ER with Orai1(CRACM1) and transient receptor potential canonical (TRPC) channel proteins in the plasma membrane (PM). This interaction is enabled by microtubular-directed reorganization of ER to form ER/PM contact points or "punctae" in which STIM1 and channel proteins colocalize. The ensuing influx of Ca^{2+} replenishes Ca^{2+} stores and sustains elevated levels of cytosolic Ca^{2+} ions-the obligatory signal for mast-cell activation. In addition, the signal can acquire spatial and dynamic characteristics (e.g., calcium puffs, waves, oscillations) that encode signals for specific functional outputs. This is achieved by coordinated regulation of Ca^{2+} fluxes through ATP-dependent Ca^{2+}-pumps and ion exchangers in mitochondria, ER and PM. As discussed in this chapter, studies in mast cells revealed much about the mechanisms described above but little about allergic and autoimmune diseases although studies in other types of cells have exposed genetic defects that lead to aberrant calcium signaling in immune diseases. Pharmacologic agents that inhibit or activate the regulatory components of calcium signaling in mast cells are also discussed along with the prospects for development of novel SOCE inhibitors that may prove beneficial in the treatment inflammatory mast-cell related diseases.

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INTRODUCTION

The generation of a calcium signal is critical for activation of mast cells and blood basophils. This signal results from receptor-mediated activation of phospholipase (PL) C and the associated production of inositol 1,4,5-trisphosphate (IP_3) which induces release of Ca^{2+} from stores in the endoplasmic reticulum (ER) and Golgi through Ca^{2+}-conducting IP_3-receptors (IP3R). Depletion of the Ca^{2+}-stores activates influx of external Ca^{2+}, a process commonly referred to as store-operated calcium entry or SOCE (reviewed in reference 1). One well characterized mechanism of Ca^{2+} influx via SOCE is the Ca^{2+}-selective calcium-release activated calcium current (I_{CRAC}) first described in RBL mast cells by Hoth and Penner.[2,3] The influx of Ca^{2+} allows reuptake of Ca^{2+} into ER through a sarco/endoplasmic Ca^{2+}-ATPase (SERCA) pump and thereby replenishes ER stores and sustains increases in concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$), the essential signal for mast cell activation. $[Ca^{2+}]_{cyt}$ is also dynamically regulated by extrusion of Ca^{2+} from the cell by a Ca^{2+}-ATPase (PMCA) pump and ion-exchange transporters in the plasma membrane. The dynamics and spatial configuration of the calcium signal within the cell is further shaped by mitochondria which possess specialized mechanisms for Ca^{2+}- uptake and efflux and a high capacity for calcium-buffering (Fig. 1). The spatial and temporal characteristics of the calcium signal, which may include calcium "puffs", "waves" and "oscillations", are thought to encode sub-signals for different cellular functions.

In addition to IgE-specific antigens, other mast cell stimulants also induce PLC-mediated calcium signals. These stimulants include the growth factor KIT ligand, also referred to as stem cell factor (SCF),[4] as well as agonists of G protein-coupled receptors such as adenosine,[5-7] PGE_2,[8,9] the formyl peptide fMetLeuPhe,[10] platelet activating factor,[10] and C3a/C5a.[11] Some mast cell activating ligands such as microbial Toll-like receptor (TLR) ligands and IL-33 can elicit modest production of cytokines without mobilizing calcium or causing degranulation but this production of cytokines is markedly enhanced by costimulation of cells with antigen which elicits synergistic costimulatory signals though calcium.[12] Calcium signals thus enable degranulation,[13,14] activation of PLA_2 for the production of the eicosanoids,[15] activation of the Ca^{2+}/calcineurin/nuclear factor of activation of T cells (NFAT) pathway for transcription of cytokine genes,[12] or Ca^{2+}-dependent cytoskeletal remodeling for chemotaxis (Fig. 1).[11]

The description of I_{CRAC} prompted a prolonged search for an elusive I_{CRAC} channel protein (CRAC). The essential components of CRAC were identified only recently as the Ca^{2+}-channel protein Orai1 (also known as CRACM1) and its activating ER calcium-sensor, stromal interaction molecule-1 (STIM1). Before then, the nonselective Ca^{2+}-conducting transient receptor potential canonical (TRPC) proteins were initially considered as candidates for CRAC but none of the TRPCs exhibited the exact characteristics or Ca^{2+}-selectivity of I_{CRAC}. Because expression of both STIM1[16,17] and Orai1[18-20] reconstituted I_{CRAC} in all fidelity[21-23] in various types of cells including mast cells, the pendulum of thinking has swung towards the notion that the activating interaction of STIM1 with Orai1 is the underlying mechanism for SOCE. There remains, nevertheless, an accumulating body of evidence that TRPCs participate in SOCE, possibly cooperatively with STIM1 and Orai1, in mast cells and other types of cells.[1] As discussed in the next section, the participation of TRPCs would be consistent with observations several decades ago that other divalent metal ions are taken up by and can substitute for Ca^{2+} in the activation of mast cells.

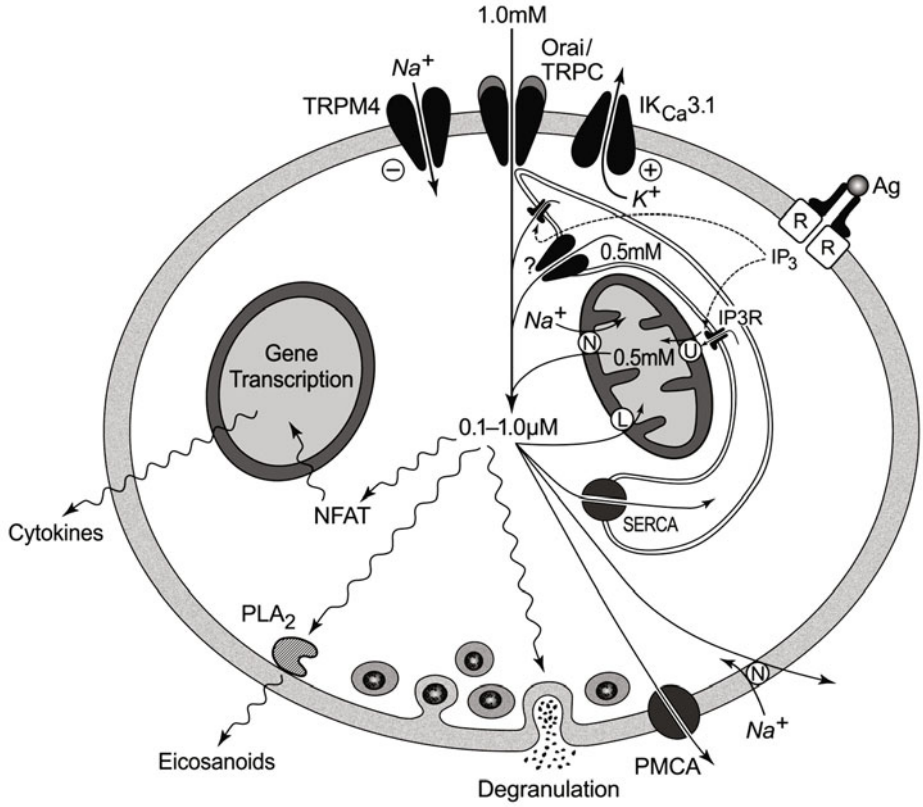

Figure 1. Calcium fluxes in stimulated mast cells. An initial event is the release of Ca^{2+} through IP_3 receptors (IP_3R) from Ca^{2+} stores in ER (as depicted), Golgi and nuclear membrane (not depicted) into the cytosol following FcεRI (R) aggregation by antigen (Ag) and the associated production of IP_3 by PLCγ. Ca^{2+} released in close proximity to mitochondria is rapidly taken up through the mitochondrial uniporter (U) which operates only with high $[Ca^{2+}]_{cyt}$ (1–10 μM) in restricted spaces (as shown) or during large oscillations in $[Ca^{2+}]_{cyt}$. In this manner, mitochondria can accelerate depletion of Ca^{2+} stores and modify the profile of the calcium signal. Elsewhere newly released Ca^{2+} diffuses through the cytosol to elevate $[Ca^{2+}]_{cyt.}$. Letm1 Ca^{2+}/H^+ antiporter (L) allows slow entry of Ca^{2+} into mitochondria at relatively low $[Ca^{2+}]_{cyt}$ (>100 nM) and thus enables mitochondria to sense small increases in $[Ca^{2+}]_{cyt}$ as a signal for increased mitochondrial activity. Letm1 is bidirectional and can extrude Ca^{2+} along with a Na^+/Ca^{2+} exchanger NCX (N) when the mitochondrial Ca^{2+}-load is high. Depletion of the Ca^{2+} stores causes formation of contact points (punctae) that bring the ER Ca^{2+}-sensor STIM1 (not shown but see Fig. 2) and channel proteins (Orai and TRPC) into close proximity to activate Ca^{2+} influx. This influx replenishes ER Ca^{2+} stores via the ATP-dependent SERCA pumps (SERCA 2b or SERCA3), sustains elevation of $[Ca^{2+}]_{cyt}$ and continuous recycling of Ca^{2+} as long as IP_3 is produced. Influx of Ca^{2+} via Orai or TRPC is negatively regulated by influx of Na^+ via TRPM4 and positively regulated by efflux of K^+ via $IK_{Ca}3.1$ which, respectively, depolarize or repolarize the membrane potential. $[Ca^{2+}]_{cyt}$ is held in dynamic equilibrium by extrusion of Ca^{2+} through the Na^+/Ca^{2+} exchangers (N) as well as the ATP-dependent PMCA pump. Candidates for a putative "leak" channel (?) in ER are discussed in the text. Also shown are the responses evoked by calcium signaling in mast cells.

In this chapter, we first describe the early studies of calcium and mast cells for reasons just noted. Subsequent sections provide descriptions of various organelles that regulate calcium signaling in mast cells. Although the contributions of ER, mitochondria and the plasma membrane are described separately for convenience of narrative, it should be emphasized that the final signature of calcium signal is determined by the coordinate actions of these three organelles (Fig. 1). The final and more speculative sections describe the functional outputs encoded by the calcium signal, the possible relevance of genetically-related defects in calcium signaling to mast cell related diseases and novel SOCE inhibitors developed for the purpose of treating allergic and autoimmune diseases.

EARLY AND RECENT OBSERVATIONS ON CALCIUM HOMEOSTASIS IN MAST CELLS: THE UNRESOLVED ISSUES

Mast cells and basophils were among the first experimental models to be used for the study of Ca^{2+}-dependent secretion when it was discovered that extracellular Ca^{2+} was required for anaphylactic release of histamine from human leukocytes,[24] rabbit basophils,[25] and rat peritoneal mast cells.[26] By then, histamine was shown to be a constituent of granules secreted from mast cells.[27] Subsequent studies revealed that Sr^{2+} or Ba^{2+} could substitute for Ca^{2+} in supporting histamine release from rat peritoneal mast cells[26,28] and that uptake of $^{45}Ca^{2+}$ and $^{89}Sr^{2+}$ correlated with the extent of this release.[28,29] Such release, whether supported by Ca^{2+} or Sr^{2+}, was effectively blocked by low concentrations of La^{3+}.[30,31] The conclusions drawn from these studies was that mast cell degranulation was dependent on substantial influx of Ca^{2+} through "Ca^{2+} channels" which can convey Sr^{2+}.[28]

Later studies with Ca^{2+}-sensitive fluorescent probes and $^{45}Ca^{2+}$ in cultured RBL-2H3 cells indicated that degranulation was absolutely dependent on an increase in $[Ca^{2+}]_{cyt}$ from ~0.1 to ~1 μM[13] through release of Ca^{2+} from intracellular stores[32-35] and influx of extracellular Ca^{2+}.[13] As in the earlier studies, this influx permitted entry of Sr^{2+} and other divalent metal ions.[36] Influx was accompanied by substantial uptake of Ca^{2+} into IP_3-sensitive and mitochondrial pools[37,38] wherein intracellular Ca^{2+} increased from approximately 0.5 mM to about 2.5 mM which then decreased as $[Ca^{2+}]_{cyt}$ subsided.[32] Influx was dependent on maintenance of the polarity of the plasma membrane, presumably through efflux of K^+,[32,39] and was counterbalanced by extrusion of Ca^{2+} from the cell by an ATP-dependent mechanism.[32,40]

Studies in other types of cells identified ER as the source of IP_3-releasable Ca^{2+} and IP_3R[41] as the channel for Ca^{2+}-release.[42] Reuptake of Ca^{2+} into ER was blocked by thapsigargin which was found to be selective inhibitor of SERCA.[43,44] This action of thapsigargin results in spontaneous loss or "leakage" of Ca^{2+} from the ER store[45,46] by a still undefined mechanism. Depletion of the ER pool, whether by thapsigargin or IP_3, invariably leads to entry of Ca^{2+} into the cell. This feature led to the proposal by Putney in 1986, of "capacitative calcium entry" now generally referred to as SOCE,[47] a process that is relevant to mast cells.[38,48] As noted in the previous section, the characterization of I_{CRAC} in mast cells by Hoth and Penner[2,3] resulted ultimately in the identification of the STIM1/Orai1 as the operational components of I_{CRAC}.

Nevertheless, the high capacity of influx mechanisms for Ca^{2+} and other divalent metal ions in mast cells are contrary to the known features of I_{CRAC} or Orai proteins. We have proposed on the basis of knockdown and overexpression studies in RBL-2H3 cells that TRPC5, which can convey Sr^{2+}, interacts with STIM1 and Orai1 to enhance influx

of Ca^{2+} and, as a consequence, allow entry of Sr^{2+}.[49] An alternative proposal based on studies in RBL-2H3 cells and bone marrow-derived mast cells (BMMC) is that TRPCs, particularly TRPC1, initiate localized Ca^{2+} puffs that potentiate IP_3R-mediated Ca^{2+}-release from nearby ER stores.[50] This potentiation leads to the generation of a calcium wave and ultimately oscillations, phenomena commonly associated with SOCE. As discussed in detail later, studies in other types of cells suggest that some TRPCs, including TRPC1 and TRPC5, form complexes with Orai proteins and STIM1to create SOCE channels whose conductances (I_{SOC}) differ from those of I_{CRAC}. The role of TRPCs in calcium signaling is controversial. One view is that CRAC channels derived from STIM1 and Orai1 are the exclusive SOCE channel. The other is that STIM1, TRPCs and Orai proteins also form SOCE channels with varying degrees of selectivity (e.g., refs. 51,52). This chapter reflects our opinion that the latter mechanism operates in mast cells.

INITIATION OF THE CALCIUM SIGNAL VIA PLC, IP_3R AND SPHINGOSINE KINASES (SKs)

Receptor-Mediated Activation of PLCγ or PLCβ Results in SOCE

A role for PLC was evident from early studies with RBL-2H3 cells in which correlations were noted in the production of inositol phosphates, increase in $[Ca^{2+}]_{cyt}$ and degranulation.[53] The PLC isoforms involved differed according to the type of stimulant; PLCγ with antigen[54,55] and PLCβ with ligands to G protein-coupled receptors.[10,56] Stimulation through either FcεRI or G protein-coupled receptors resulted in release of Ca^{2+} from a common IP_3/thapsigargin-sensitive pool which was assumed to be in ER.[5,38,57] Recently, stochastic modeling using three-dimensional constructs of electron-microscopic images of RBL-2H3 cells, along with supporting data, fits well with the concept of release of Ca^{2+} from ER through activation of IP_3Rs by IP_3.[58]

RBL-2H3 cells, BMMC and human peripheral blood-derived mast cells express both $PLC\gamma_1$ and $PLC\gamma_2$.[59-61] $PLC\gamma_1$ resides primarily in cytosol of RBL-2H3 cells but then migrates to the plasma membrane where it localizes largely in actin-rich membrane ruffles after antigen stimulation.[59] $PLC\gamma_2$, in contrast, is associated with Golgi and plasma membranes and remains so after stimulation. Both isoforms are tyrosine phosphorylated and thus activated, following antigen stimulation. The pathways leading to PLCγ phosphorylation are complex and are not entirely clear. Induction of FcεRI aggregation by antigen initiates phosphorylation cascades that involve the Src kinases Lyn and Fyn as well as Syk tyrosine kinase, phosphorylation of adaptor proteins, recruitment of additional signaling molecules by these adaptors and ultimately production of IP_3 by PLCγ. The adaptor proteins, linker for activation of T cells (LAT) and SH2-containing leukocyte-specific protein of 76 KDa (SLP-76) are critical for recruitment of $PLC\gamma_1$ and calcium signaling (for a more detailed account see reference 62). Another essential component of mast cell activation is phosphoinositide 3-kinase (PI3K) whose activity is regulated largely by Fyn through the adaptor protein, Grb2-associated binder-like protein 2 (Gab2).[63] It is unclear, however, whether the PLCs are activated separately through different pathways. Differences have been noted in RBL-2H3 cells where phosphorylation of $PLC\gamma_1$, but not $PLC\gamma_2$, is suppressed by the PI3K inhibitor wortmannin.[59] In these cells, $PLC\gamma_1$ was assumed to play the predominant role as both antigen-induced IP3 production and degranulation are also inhibited by wortmannin. In BMMC and human peripheral blood-derived mast cells, the

initial activation of $PLC\gamma_1$ occurs independently of PI3K.[61] Indeed the phosphorylation and activation of both $PLC\gamma_1$ and $PLC\gamma_2$ are resistant to wortmannin, are still apparent in BMMC that lack p85 PI3K subunits and appear to precede the activation of PI3K. Wortmannin partially inhibits degranulation leaving the possibility that PI3K mediates later signals for degranulation.[61] The differential distribution of the two isoforms of PLCγ in RBL-2H3 cells could result theoretically in calcium signals with different signatures. Whether the PLC isoforms are regulated differently and play complimentary roles in calcium signaling in mast cells are issues that require further investigation.

The pattern of expression of PLCβ isoforms in mast cells varies. Of the three known PLCβ isoforms, only PLCβ3 is expressed in RBL-2H3 cells[56,64] whereas $PLC\beta_2$ and $PLC\beta_3$ are expressed in BMMC.[9] PLCβ is activated through receptors that engage either the pertussis-sensitive G protein, G_i,[9] or the pertussis- and cholera- toxin insensitive G protein, G_q.[6] Receptors that operate through G_s and adenylate cyclase are generally inhibitory. Some of the $G_{i/q}$-linked receptors undergo rapid desensitization such that IP_3 production and calcium signal are transient and insufficient to promote functional responses.[8,65] However, ligands to such receptors can markedly potentiate the functional responses to antigen because of synergistic signaling interactions provoked by the sustained calcium signal induced by antigen.[65]

Role of the Sphingosine Kinase (SK) Isoforms, SK1 and SK2

Sphingosine was first identified and so named by Thudichum in 1884 as a metaphor for the mythological riddle of the Sphinx.[66,67] In some respects this is still true for calcium signaling.[1,68,69] In the modern era, the SK product, sphingosine 1-phosphate (S1P), was proposed to complement the actions of IP_3 in promoting SOCE, degranulation,[70,71] and the production of eicosanoids and cytokines[72] in mast cells. Both IP_3 and S1P were considered essential for release of Ca^{2+} from intracellular stores[70] with S1P promoting transient release and IP_3 a more sustained release coupled to Ca^{2+}-entry.[71] However, a subsequent report suggests that S1P regulates primarily Ca^{2+}-entry rather than release.[72] Another ambiguity is that SK1 was claimed to be the predominant regulator of calcium signaling in one study[71] and SK2 in another.[72] An uncertainty is that S1P is released from stimulated mast cells[73] and could act in an autocrine manner[74] to increase $[Ca^{2+}]_{cyt}$ via PLCβ-coupled S1P receptors.[75] Potential upstream regulators of the SK/Ca^{2+}-pathway include the Src kinases Lyn and Fyn,[76] PLD1 and clathrin which is thought to facilitate transfer of the lipophilic S1P from the plasma membrane to ER.[77] Unlike IP_3, S1P is not freely diffusible in the cytosol[68] and the Ca^{2+}-channel(s) targeted by S1P have yet to be identified.[69] Regardless of these unresolved issues, there is corroborative and extensive evidence that S1P and related sphingolipids are critical for mast-cell driven allergic reactions[74,78-80] as discussed in detail in the Chapter 8 in this book.

Function and Regulation of IP3R in ER

IP3Rs are of three subtypes (designated 1, 2 and 3) and are expressed predominantly in the ER and to a lesser extent in nuclear reticulum (NR) and Golgi.[81-85] The IP3Rs are usually restricted to particular subcellular locations. In RBL-2H3 cells, IP3R1 and IP3R2 are located in ER and NR whereas IP3R3 is located elsewhere.[86,87] RBL-2H3 cells express ~14,000 tetrameric IP3Rs with IP3R2 being the predominant form (70% of total) and IPR1 and IPR3 constituting 10% and 20% of total IP_3Rs.[87] As in other types

of cells,[81] stimulation of RBL-2H3 cells results in redistribution of IP_3R from a diffuse to clustered pattern in ER and NR.[87] The relevance of clustering is unclear but it could signify association with sites of IP_3 production[58] or formation of signaling complexes, sometimes referred to as signalosomes, which may contain IP_3R, TRPCs, receptors for activated C-kinase-1 (RACK1), STIM1 and Orai1 (see ref. 88 and citations therein).

IP_3Rs form functional Ca^{2+} channels only after oligomerization to form homo- or hetero-tetramers.[85,89] Interestingly, the same is true for Orai and TRPC proteins which also form functional channels only after tetramerization. Each IP_3R subunit consists of a cytosolic N-terminal IP3-binding domain, a large intermediate modulatory domain and a C-terminal helical transmembrane-spanning domain which, in the tetrameric state, form part of the Ca^{2+} channel. The IP_3-binding domain lies in close proximity to the channel pore to enable conformational changes that are sufficient for channel gating.[85,89] The intermediate domain contains binding sites for Ca^{2+}, calmodulin and other factors that regulate $IP3_R$ function.[81,83,85] All three IP_3Rs exhibit biphasic responses to Ca^{2+} in which IP_3R activity is enhanced or diminished at low or high $[Ca^{2+}]_{cyt}$ respectively[90] by mechanisms that are still conjectural.[1] Other regulatory factors include RACK1 and scaffolding proteins that may allow targeting of IP_3R to strategic sites and formation of signaling complexes.[83,85] IP_3Rs are phosphorylated by multiple serine/threonine protein kinases creating potential positive feed-back loops to enhance Ca^{2+}-release.[85,91] The Src tyrosine kinases, Fyn in T-cells[92] and Lyn in B cells,[93] also phosphorylate and positively regulate IP_3R but it is not known if this occurs upon activation of these same kinases in mast cells. Many aspects of the regulation of IP_3R activity remain unclear as described in several recent reviews.[83,84,89,91]

IP_3-SENSITIVE INTRACELLULAR Ca^{2+}-STORES AND COUPLING TO SOCE CHANNELS

IP_3-Releasable Ca^{2+} Stores

In addition to ER, IP3-releasable Ca^{2+} stores are present in NR[94] and Golgi.[95] The ER and NR form a contiguous Ca^{2+}-pool of ~0.5 mM but Golgi forms a separate pool of ~0.3 mM Ca^{2+}. Initial increases in $[Ca^{2+}]_{cyt}$ are primarily due to release of Ca^{2+} from ER and Golgi whereas release from NR is largely confined to the nuclear space.[94] All three structures contain the thapsigargin-sensitive SERCA pump that ensures recapture of cytosolic Ca^{2+} against a high concentration gradient (also see following section). The Golgi membranes contain, in addition, a thapsigargin-insensitive Ca^{2+}-ATPase (SPCA) pump. The individual contributions of Golgi and ER to the calcium signal in mast cells are unknown but several IP_3-releasable pools are discernable in RBL-2H3 cells.[96] Two of these are thapsigargin-sensitive of which only one is directly linked to I_{CRAC}. The other is a thapsigargin-insensitive store which is presumed to be dependent on a calcium ATPase other than SERCA for refilling of this Ca^{2+} store. Compartmentalization of Ca^{2+} stores in ER/Golgi is also suggested from the differential effects of IP_3, thapsigargin and ionomycin on I_{CRAC} dynamics in the RBL1 mast cell line.[97] The nature of these pools or compartments is unclear but there is evidence that, in some types of cells, Ca^{2+} can diffuse throughout the lumen of ER and depletion of the entire ER store is required for SOCE whereas in other types, such as polarized liver cells, diffusion of Ca^{2+} is restricted and depletion of a limited region of ER is sufficient to activate SOCE.[98] It is likely that

SOCE is activated in RBL-2H3 cells by depletion of ER stores near the cell periphery as SOCE can be activated by just 1 nM thapsigargin,[38] a concentration that is known to localize and act only in regions of ER in close proximity to the plasma membrane.[99] This concentration of thapsigargin does not induce degranulation whereas more conventional concentrations of thapsigargin (e.g., 1 μM) produce a global depletion of ER stores and degranulation.[38,99]

Actin and microtubular interactions direct ER motility which is essential for regulation of calcium signaling and other cellular functions.[100] This motility, as we shall discuss later, permits functional contacts of ER with Ca^{2+} channels in the plasma membrane. Depolymerization of microtubules or inhibition of the microtubular motors, kinesin or dynein, reduces SOCE.[101,102] Unintended effects of pharmacologic agents on ER motility and calcium signaling should be considered in studies of mast cell activation and function.

Ca^{2+} Uptake and "Leakage" in ER

Uptake and "leakage" of Ca^{2+} across the ER membrane appear to be dynamically balanced in resting RBL-2H3 cells.[103] The "Ca^{2+} leak" from ER is apparent by a relatively slow increase in $[Ca^{2+}]_{cyt}$ in the absence of external Ca^{2+} following blockade of SERCA by thapsigargin. Subsequent provision of external Ca^{2+} results in a rapid and substantial increase in $[Ca^{2+}]_{cyt}$ which is the classic hallmark of SOCE. The number of SERCA channels, as determined by thapsigargin binding, has been estimated as 1.6 million/ RBL-2H3 cell.[103] Of the three known SERCA proteins and their spliced variants, only SERCA2b and SERCA3 are found in mast cells[104] where they codistribute with IP_3R1, IP_3R2 and calreticulin on density gradients.[86] IP_3R3 is the anomaly in that it does not appear to be associated with ER or the SERCAs.[86] The SERCA proteins transport two Ca^{2+} ions for each ATP molecule consumed which permits efficient recapture of released Ca^{2+} from the cytosol and possibly ensures refilling of ER with minimal perturbation of $[Ca^{2+}]_{cyt}$ when ER is in close proximity to SOCE channels.[105] SERCA activity also enables Ca^{2+} oscillations in cells.[106] The SERCAs are regulated directly by free Ca^{2+} within ER.[107] The Ca^{2+} sensing mechanism, however, is uncertain but suggested mechanisms include interactions with calreticulin and calnexin,[108] STIM1,[109,110] and presenilins.[111] Calreticulin and calnexin reside in the ER lumen and ER membrane respectively and are interacting proteins that are best recognized for their role in protein folding. Both proteins as well as the phosphorylation of calnexin are critical for maintenance of SERCA-dependent Ca^{2+} oscillations in *Xenopus* oocytes.[108,112] It is unknown whether similar mechanisms exist in mast cells but it is notable that calnexin is prominently phosphorylated in antigen-stimulated RBL-2H3 cells.[113]

The nature of the ER "Ca^{2+}-leak" protein is still a matter of debate (hence the question mark in Fig. 1). Proposed candidates include the presenilins,[114,115] the translocon complex,[116-118] and pannexin-1.[119] The presenilins were reported to form Ca^{2+}-permeable channels in bilayer vesicles whereas mutant presenilins that are thought to be responsible for enhanced calcium signaling in Alzheimer's disease lacked such activity.[114,115] However, others report no abnormality in ER Ca^{2+} "leakage" in cells from such patients but have noted enhanced IP_3-mediated release.[120] The aberrant calcium signaling in Alzheimer's disease has been attributed to interactions of constitutively active mutant presenilins with IP_3Rs.[121] The evidence in total does suggest a role for the presenilins in regulating Ca^{2+} homeostasis in part because of the links to Alzheimer's disease and the presumed interactions of presenilins with SERCA[111] and IP_3R.[122] Although presenilins

are expressed and possibly contribute to calcium signaling in mast cells, their role has yet to be determined.[1] Translocon, a protein-conducting channel in ER, can serve as a "leak pathway" for Ca^{2+}[117,123] and can activate SOCE[116,124] when not engaged in protein synthesis.[125] However, contrary data has led to the conclusion that it is normally closed and is irrelevant to the physiological calcium leak mechanisms.[118] The pannexins, a recently described family of ubiquitous molecules analogous to gap junction connexins, form not only intercellular gap junction channels but also Ca^{2+}-permeable channels in ER. Overexpression and knockdown studies implicate pannexin1 as a mediator of "Ca^{2+}-leak" and an activator of SOCE "in addition to any other potential leak mechanism."[119] Whether one or more mechanisms actually exist, leakage has the intrinsic properties of an ion channel by exhibiting monoexponential efflux of Ca^{2+} from ER which shuts off once calcium stores have reached about 7% of their normal level.[126] Leakage is also in dynamic balance with ER uptake.[58]

The ER Ca^{2+}-Sensors, STIM1 and STIM2

The absolute requirement for STIM1 and Orai1 for activating I_{CRAC}, calcium signaling, degranulation and cytokine production in mast cells has been demonstrated in BMMC derived from STIM1 or Orai1 deficient mice.[127,128] These mice also have attenuated IgE-dependent allergic responses. Both isoforms of STIM are known regulators of SOCE in a variety of cell types.[16,17,20] Both isoforms are highly homologous and contain an N-terminal EF hand along with a sterile α-motif (SAM) located in the ER lumen, a single transmembrane domain and protein-interacting domains in their lumenal and cytoplasmic portions.[16,17,129] The affinity of the EF-SAM domains for Ca^{2+} is appropriately set to sense the concentration of free Ca^{2+} in ER ($[Ca^{2+}]_{ER}$) and state of depletion of ER Ca^{2+}-stores.[130,131]

When ER Ca^{2+} stores are full, STIM1 is diffusely distributed throughout the ER microtubular network[101] but as these stores are depleted, STIM1 oligomerizes and migrates to peripheral ER "punctae" in close proximity to the plasma membrane where it can activate I_{CRAC}.[16,22,132] Mutating the STIM1 EF-hand results in constitutive localization of STIM1 in "punctae" and activation of I_{CRAC} independently of store-depletion.[132,133] Collectively these studies imply that on store depletion dissociation of Ca^{2+} from the EF-hand, which is mimicked by mutation of the EF-hand, results in oligomerization and relocation of STIM1. Rearrangement of ER with formation of "punctae" containing colocalized STIM1 and Orai1 is also evident in antigen-stimulated RBL-2H3 cells.[134] Maximal colocalization coincides with the initial spike in $[Ca^{2+}]_{cyt}$ with rapid reversal upon decay in $[Ca^{2+}]_{cyt}$ and refilling of ER stores.

The generally accepted scenario from studies in RBL-2H3[134-136] and other types of cells is that coiled-coil domains in STIM1 and Orai1 and the electrostatic interactions between these two molecules are critical for the association of these two molecules and the activation of I_{CRAC}.[1] The oligomerization and migration of STIM1 to the cell periphery is dependent on a coiled-coil and another domain (aa 233-450, human numbering) in the cytoplasmic C-terminal region of STIM1.[135,137] Functional interaction with Orai1 is dependent on a minimal domain (aa 342-448) in STIM1 called the CRAC activating domain (CAD)[138] which contains a short conserved basic sequence (aa 382-387) that is sufficient to activate SOCE in RBL-2H3 cells.[136] It is postulated that neutralization of a C-terminal acidic coiled-coil domain of Orai1[134,139] by CAD transmits a gating signal for SOCE. In support of this idea, amphiphilic molecules such as D-sphingosine and ***N,N***-dimethylsphingosine inhibit not

only IP_3-mediated I_{CRAC}[140] but also the FRET-monitored interaction of STIM1 with Orai1 and Ca^{2+}-influx.[134] The proposed model was that the positively charged sphingosines, which flip to the cytoplasmic surface of the plasma membrane, neutralize the Orai1 acidic residues resulting in Orai1 homo-oligomerization and preclusion of STIM1. Although these findings may point to regulation of SOCE by sphingolipids, they exclude a direct role for SKs because D-sphingosine is a substrate and ***N,N***-dimethylsphingosine is an inhibitor of SK, yet both have the same effect.

STIM1 also interacts with various TRPC channels, either individually or as heteromeric combinations with Orai,[52,141,142] and may do so though intramolecular electrostatic interactions between complementary positively-charged residues in STIM1 and negatively-charged residues in TRPC1 or TRPC3. However, it is claimed that the coupling mechanisms for Orai and TRPC are different.[142]

STIM2 has not been studied as extensively as STIM1 and not at all in mast cells. The STIM isoforms differ markedly in their rates of oligomerization and dissociation which may reflect differences in their regulation of calcium signaling.[143] The indications are that STIM2 tightly regulates basal $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{ER}$ as STIM2 migrates to peripheral punctae and activates Orai1 in response to relatively small decreases in $[Ca^{2+}]_{ER}$.[129] In contrast, STIM1 comes into play with more profound store depletion as indicated in cells from mice with conditionally targeted alleles of *STIM1* and *STIM2*.[144] Deficiency of STIM1 severely impairs thapsigargin-induced SOCE in these cells whereas deficiency in STIM2 had much smaller effects. One model based on studies with ectopic STIM proteins and whole-cell dialysis views STIM2 as constitutively active under basal conditions and negatively regulated by high $[Ca^{2+}]_{cyt}$.[145]

MECHANISMS OF Ca^{2+} ENTRY (SOCE) AND EXPORT ACROSS THE PLASMA MEMBRANE

Ca^{2+} Entry via TRPC and Orai Proteins

TRPCs are a subset of a superfamily of TRPs which include TRPC, TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin) and TRPML (mucolipin).[146] All seven members of the TRPC subfamily have been investigated as potential candidates for CRAC channels and certain TRPMs are thought to negatively regulate plasma membrane polarity which, as we discuss later, is essential for maintaining SOCE activity.

As noted earlier, none of the TRPCs exhibit the exact characteristics of I_{CRAC}[147,148] In particular, some TRPC channels conduct Sr^{2+} and other divalent metal ions in addition to Ca^{2+} and have single channel conductances several orders of magnitude greater than I_{CRAC}. All TRPCs are activated as a consequence of activation of PLC, either through store depletion or the generation of diacylglycerol.[146] Formation of a functional cation channel requires assembly of four TRPCs either as homomeric or heteromeric tetramers such as TRPC1 with TRPC3, TRPC4, TRPC5, or TRPC7 and TRPC3 with TRPC6 or TRPC7.[149] The electrophysiological characteristics vary significantly according to the constituents of the complex.[146] Each TRPC monomer consists of six transmembrane segments (S1-S6) with the N- and C-terminal regions located within the cell. The pore-forming loop between S5 and S6 form part of the ion channel. The TRPCs contain, in addition, several consensus binding domains in the C-terminal portion and several ankyrin repeats in the N-terminal

region. Both the C- and N- terminals contain coiled-coil domains which are thought to be essential for oligomerization.[150]

Like $IP3_R$s, TRPCs interact with accessory proteins to form signaling complexes.[149] The accessory proteins are thought to regulate the cellular destination and function of the TRPCs. They include proteins involved in cytoskeletal interactions, vesicular trafficking and calcium signaling such as calmodulin, immunophillins, PLC, IP_3R, STIM1, PMCA and SERCA.[149-151] The ankyrin regions of TRPC appear to account for strategic targeting in the plasma membrane. Some TRPCs appear to be positively regulated by IP_3R through interaction with an IP_3R consensus binding-domain in TRPC. One concept is that TRPCs not only mediate SOCE but, in conjunction with IP_3Rs, also regulate the amplitude of the calcium signal in localized regions for specialized cellular functions.[149,152]

The Orai proteins are atypical cation channel with four transmembrane domains.[18-21,23] Three mammalian *Orai* gene products have been identified in a wide variety of cells. All three contain a proline/arginine-rich region in the N-terminus and a putative C-terminus coiled-coiled domain.[153] The selectivity filter of Orai1 is linked to acidic residues in the first and third transmembrane domains and the first loop segment. As noted, formation of a functional CRAC channel requires formation of tetramers of Orai1.[154,155] Orai1 can also oligomerize with Orai2 and Orai3 to create CRAC channels each exhibiting modest differences in ion-selectivity and inhibition by elevated $[Ca^{2+}]_{cyt}$.[156] These differences could provide additional flexibility to the regulation of calcium signaling but their physiological relevance has not been demonstrated. All three Orai proteins can be activated by STIM2 as well as by STIM1.[145]

Orai1 along with STIM1 were recognized as the elusive components underlying I_{CRAC} through several lines of evidence.[16-20] Most dramatic was the demonstration that a mutation of *Orai1* was linked to a severe combined immune deficiency (SCID) that was associated with deficient Ca^{2+}-influx in T cells. This deficiency could be rectified by expression of wild type Orai1.[18] Overexpression of Orai1 and STIM1 together, but not individually, in a variety of cells resulted in substantial I_{CRAC}.[21-23] The field is evolving rapidly and the reader should consult recent reviews on the subject,[148,157-160] some focused exclusively on immunological cells,[161-164] for more detailed information. We have already noted that STIM1 and Orai1 are absolutely essential for activation of mast cells in vitro and in vivo.[127,128] Nevertheless, the properties of I_{CRAC} and Orai proteins would not account for the permeability of mast cells to divalent metal ions and Ca^{2+} or the nonselective cation currents (I_{SOC}) that have been recorded in activated RBL-2H3 cells.[165,166] The explanation, we believe, is the interaction of less selective TRPC channels with Orai1 and STIM1. As noted earlier, knockdown of endogenous TRPC5, STIM1, or Orai1 individually with inhibitory RNAs substantially reduces influx of Ca^{2+} as well as degranulation in RBL-2H3 cells.[49] Moreover, overexpression of Orai1 with STIM1 promotes constitutive influx of Ca^{2+} but not of Sr^{2+} whereas overexpression of TRPC5 with STIM1 promotes constitutive influx of both ions. Sr^{2+} is used here only as a diagnostic probe as it is physiologically irrelevant. The data suggest that Sr^{2+}-permeable TRPC5 acts coordinately with Orai1 and STIM1 to allow Sr^{2+} to permeate and induce degranulation (Fig. 2).[49]

In support of the view that TRPCs participate in SOCE, studies in various types of cells have demonstrated that Orai1 forms complexes with some TRPCs and in conjunction with STIM1 creates currents (I_{SOC}) with properties distinct from I_{CRAC}.[49,167-169] Such interactions include TRPC1 or TRPC5 with Orai1[49,51,167,169] and TRPC3 or TRPC6 with Orai1, Orai2, or Orai3.[168] Apart from electrophysiological recordings and ablation of SOCE currents by use of siRNA technology, there are no pharmacological diagnostic probes. Like I_{CRAC},

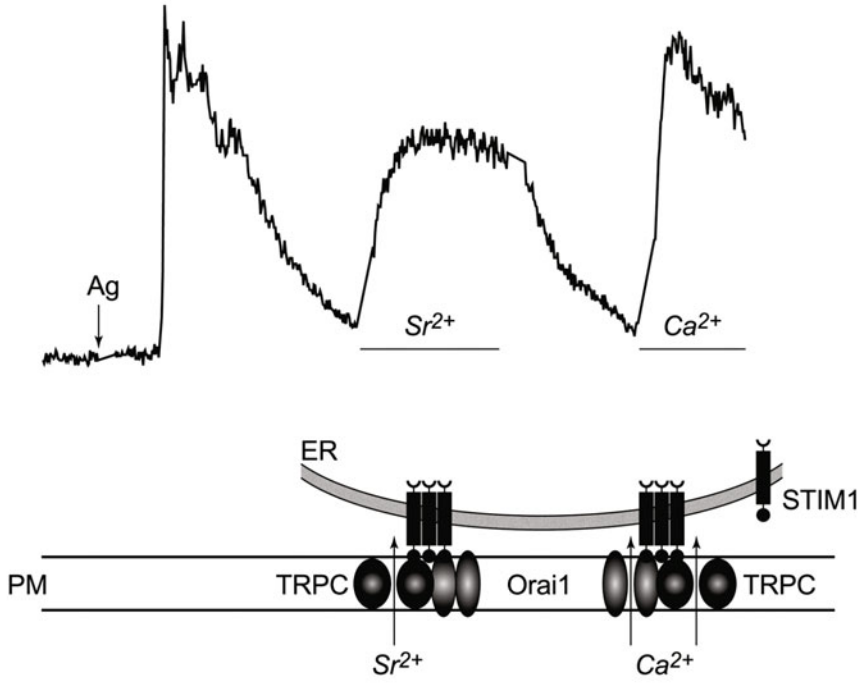

Figure 2. Antigen-induced entry of Sr^{2+} and Ca^{2+} via a STIM1/Orai1/TRPC complex. Changes in cytosolic Ca^{2+} and Sr^{2+} were monitored by single cell imaging of fura 2-loaded RBL-2H3 cells. Cells were stimulated with antigen (Ag) in Ca^{2+}-free medium except for the periods indicated by the bars when either 3 mM Sr^{2+} or 1 mM Ca^{2+} were included in the medium. The trace reflects release of Ca^{2+} from intracellular stores (1st peak), entry of Sr^{2+} (2nd peak) and of Ca^{2+} (3rd peak). The model proposed is that on depletion of ER stores and dissociation of Ca^{2+} from STIM1, STIM1 ologomerizes, translocates to ER/plasma membrane junctions and interacts with a TRPC5 and Orai1 complex to activate entry of Sr^{2+} (via TRPC5) and Ca^{2+} (via TRPC5 and Orai1).[49] Similar results have been obtained in BMMC and human mast cells derived from peripheral blood.

I_{SOC} is blocked by low concentrations (1 μM) of La^{3+} and 2-aminoethoxydiphenyl borate (2-APB).[51] If Orai proteins interact not only among themselves but also with TRPCs this would add further flexibility to the calcium "tool kit" that is available to cells. The future challenge, nevertheless, is to demonstrate these various combinatorial arrangements actually result in specific outcomes.

Polarity of Plasma Membrane Regulates Ca^{2+}-Entry

Although Ca^{2+}-influx in mast cells is driven by the high concentration gradient of Ca^{2+} across the plasma membrane (about 1/10,000), it is also dependent on maintenance of the electrochemical polarity of the plasma membrane.[170] Depolarization of the plasma membrane with high external K^+ abolishes Ca^{2+}-influx and degranulation.[32] The mechanisms for setting basal membrane potential may vary among subpopulations of mast cells, but the mechanism for repolarization appears similar. Resting RBL-2H3 cells express an inwardly rectifying K^+ channel, Kir2.1, which sets the membrane potential at about -80

mV[171] whereas resting primary human cell lines have a membrane potential of about 0 mV with no demonstrable Kir current.[172] All these cell lines depolarize following influx of Ca^{2+} and then repolarize by a mechanism attributed to K^+ efflux through the Ca^{2+}-activated $iK_{Ca}3.1$ (also known as $iK_{Ca}1$) potassium channel (Fig. 1).[172-174] Blockade of $iK_{Ca}3.1$ impairs degranulation[174] and chemotaxis.[175] The presence of stem cell factor (SCF), interleukin (IL)-6, or IL-10 increases the expression of functional $iK_{Ca}3.1$ in human mast cells and the authors suggest that this could account for the enhanced reactivity of these cells to antigen.[174] Chloride channels have also been implicated in regulating membrane polarity, Ca^{2+}-influx and degranulation in mast cells. The contributions of these channels to mast cell activation are not well defined and the reader is referred to a more detailed discussion of this topic in a recent review.[172]

The nonselective Ca^{2+}-activated cation channels, TRPM4 and TRPM5, are thought to be negatively regulate SOCE.[176] Both promote influx of monovalent but not divalent cations in mast cells.[177] TRPM4, in particular, has been identified as promoting membrane depolarization and limiting Ca^{2+} influx (Fig. 1) and activation of BMMC in vitro and in vivo.[178,179] This is evident from increased FcεRI-mediated Ca^{2+}-entry, release of inflammatory mediators and chemotaxis in TRPM4$^{-/-}$ BMMC as well as increased severity of the acute phase of IgE-mediated cutaneous anaphylactic response in TRPM4$^{-/-}$ mice. On this basis, TRPM4 is suggested as a potential therapeutic target for allergic diseases[178] but the prospects would depend on whether TRPM4 acts similarly in other types of cells. TM-58483, an activator of TRPM4, suppresses Ca^{2+} and cytokine production in lymphocytes.[180]

Export of Ca^{2+} from Cells via Ca^{2+}-ATPase Pumps (PMCAs) and Ion Exchangers

The orchestration of the calcium signal requires not only influx of Ca^{2+} but also its extrusion from cells. Extrusion is accomplished actively through PMCAs and passively through Na^+/Ca^{2+} ion exchangers (Fig. 1). However, because of their high conducting capacity, Na^+/Ca^{2+} exchangers probably play the predominant role in Ca^{2+} extrusion once $[Ca^{2+}]_{cyt}$ is elevated. The more metabolically demanding PMCAs may then finely tune $[Ca^{2+}]_{cyt}$ towards basal levels.[181]

PMCA shares many of the same structural and functional features as the SERCAs in ER and SPCA in Golgi. All three belong to a large family of P-type ATPases which are so named because the reaction cycle involves formation of a phosphorylated intermediate.[182] However in contrast to SERCA, the PMCAs contain an extended C-terminal tail and transport one Ca^{2+} ion for each ATP molecule consumed as opposed to two Ca^{2+} ions for SERCAs. Much has been deduced about the structure and mechanisms of these pumps from the recent determination of the crystal structure of SERCA.[182,183] Four PMCA isoforms and many of their spliced variants, have been cloned. Each PMCA contains an ATP-binding domain and a critical aspartate residue which is phosphorylated during each cycle of Ca^{2+} transport. This phosphorylation cycle is regulated by a Ca^{2+}/calmodulin-binding region in the C-terminal domains of the PMCAs and is inhibited at low $[Ca^{2+}]_{cyt}$ and facilitated when $[Ca^{2+}]_{cyt}$ is elevated.[1] As is the case for IP_3R and SERCA, PMCAs form multiprotein complexes within specialized membrane domains and, in this manner, PMCA activity may be modulated by protein kinases and acidic phospholipids particularly the inositol phospholipids, sphingosine and phosphatidyl serine.[181,184] PMCAs can adapt rapidly to CRAC[185] and other Ca^{2+} channel activities in the cell.[181] PMCAs are concentrated in strategic locations such as the apical membranes of pancreatic and

salivary gland cells.[186,187] Microarray data suggest that human umbilical cord blood-derived mast cells express PMCA1 and PMCA4[188] but, in general, the functions of individual PMCA isoforms are not well defined. Studies in knockout mice suggest that PMCA1 is a regulator of cellular calcium in most or all cells whereas PMCA2 and PMCA4 have specialized cell-specific functions.[182]

The Na^+/Ca^{2+} exchangers not only enable rapid Ca^{2+} extrusion but may also decouple SOCE from the inhibitory effects of elevated $[Ca^{2+}]_{cyt}$. These exchangers are categorized as K^+-independent (designated as NCX) or K^+-dependent (designated as NCKX).[189] Although both families of exchangers are bidirectional and are driven by the electrochemical Na^+ and Ca^{2+} gradients across cell membranes, NCKX family members are also dependent on the K^+ gradient.[189] Under physiologic conditions the Na^+/Ca^{2+} exchangers extrude Ca^{2+} in exchange for Na^+ when $[Ca^{2+}]_{cyt}$ is elevated but ion exchange can be reversed by low concentrations of external Na^+. At normal external concentrations of Na^+, the exchangers positively regulate influx of Ca^{2+} or Sr^{2+}, increase in $[Ca^{2+}]_{cyt}$ and degranulation in mast cells by minimizing Ca^{2+}-dependent inhibition of SOCE channels.[190-192] At low concentrations of external Na^+, Ca^{2+} efflux is substantially reduced[191] and Na^+/Ca^{2+} fluxes appear to be closely correlated when external concentrations of Na^+ are varied.[190] This correlation is one indication of the prominence of Na^+/Ca^{2+} exchange during mast cell activation. Whole cell patch-clamp recordings on RBL-2H3 cells and BMMC indicate that both K^+-dependent and K^+-independent Na^+/Ca^{2+} exchangers (identified as NCKX3, NCKX1 and NCX3) operate during SOCE and that they may account for as much as 50% of the Ca^{2+} extruded from cells when $[Ca^{2+}]_{cyt}$ is elevated.[192] Another indication is that the calculated maximal rate of Ca^{2+} efflux is 129 μM/s for Na^+/Ca^{2+} exchange versus 17.5 μM/s for PMCA in stimulated RBL-2H3 cells.[58] In other types of cells Na^+/Ca^{2+} exchangers operate in conjunction with PMCA and SERCA to regulate $[Ca^{2+}]_{cyt}$[192] and, like PMCA and SERCA, they interact with numerous regulators.[189] These include lipids, especially phosphatidylinositol 4,5-bisphosphate, as well as cytoskeletal, scaffolding and signaling proteins.

MITOCHONDRIA MODULATE CALCIUM SIGNALING

The dynamics of the calcium signal are also dependent on mitochondrial activity in all cells[193,194] including mast cells.[37,38] Three dimensional reconstruction of electron microscopic images of RBL-2H3 cells depict mitochondria as frequently surrounded by ER[58] to form ER/mitochondrial junctions[195] with access to ER SERCA pumps.[196] This architecture facilitates rapid uptake of newly released Ca^{2+} into mitochondria.[58] Indeed, IP_3 induces incremental and coordinated Ca^{2+}-release from ER and Ca^{2+}-uptake into mitochondria in permeabized RBL-2H3 cells.[196] In intact RBL-2H3 cells, transfer of Ca^{2+} to mitochondria is most efficient during Ca^{2+} oscillations where the peak $[Ca^{2+}]_{cyt}$ appears to activate a mitochondrial Ca^{2+} uniporter.[197] This uptake enables rapid depletion of ER stores and in some cells localization of the calcium signal to specific subcellular domains.[193] Although mitochondria are not as intimately associated with the plasma membrane as ER in RBL-2H3 cells,[58] studies in other cells suggest that mitochondria in the vicinity of CRAC channels maintain CRAC activity by minimizing localized elevations of $[Ca^{2+}]_{cyt}$ that would otherwise inhibit CRAC activity.[198,199] In polarized cells, such as pancreatic acinar cells, the positioning of mitochondria confines Ca^{2+} waves to the apical area.[200]

It was initially thought that mitochondrial uptake occurred exclusively through a mitochondrial Ca^{2+} uniporter (MCU) which operates at relatively high $[Ca^{2+}]_{cyt}$ (i.e., 1-10 μM) but whose protein composition is still undefined.[201] However, it is now apparent that a Ca^{2+}/H^+ antiporter, the leucine-zipper-EF-hand-containing transmembrane (Letm1) protein operates at much lower $[Ca^{2+}]_{cyt}$ (100 nM or lower).[202] MCU uptake is driven by a negative membrane potential maintained by the respiratory chain and is activated by calmodulin at high $[Ca^{2+}]_{cyt}$. This allows rapid uptake of Ca^{2+} particularly in the vicinity of Ca^{2+} channels and during calcium transients thus restricting increases in Ca^{2+} to specific cellular domains.[193,203] Letm1, in contrast, permits slow entry of Ca^{2+} in exchange for H^+ (1 for 1) at low $[Ca^{2+}]_{cyt}$ and its activity is dependent on the electron transport chain. Letm1 thus enables mitochondria to decode subtle changes in $[Ca^{2+}]_{cyt}$ and regulate ATP production without risk of Ca^{2+} overload and apoptosis.[194] Mitochondrial uptake and, as a consequence, SOCE are impaired in RBL-2H3 cells by the mitochondrial respiratory chain inhibitor, antimycin A, in combination with the ATP synthase inhibitor, oligomycin.[37]

Total mitochondrial calcium can reach millimolar concentrations as a freely dissociable calcium phosphate ($Ca_3(PO_4)_2$) complex although free Ca^{2+} concentrations in mitochondria ($[Ca^{2+}]_m$) remain at 0.5-2 μM regardless of total calcium-load.[204] Egress of Ca^{2+} is mediated by the Na^+/Ca^{2+} exchanger, NCX, which permits recycling of Ca^{2+} across the mitochondrial membrane.[204] Mitochondrial Ca^{2+} influx and efflux reaches rapid equilibrium over a wide range of loading conditions to buffer external Ca^{2+} at 0.5-1.0 μM, the so called mitochondrial "set-point" for $[Ca^{2+}]_{cyt}$,[205] which appears to be the case in RBL-2H3 cells.[38] Enhanced mitochondrial NCX activity in patients with human mitochondrial Type 1 deficiency results in reduced mitochondrial Ca^{2+} levels and function along with aberrant calcium signaling in cells from these patients.[206]

THE SIGNATURE OF CALCIUM SIGNALS AND FUNCTIONAL CONSEQUENCES IN MAST CELLS

Calcium signals in individual mast cells are generally apparent as a sustained increases or oscillations in $[Ca^{2+}]_{cyt}$. In RBL-2H3 cells, oscillations are of variable amplitude and duration and are usually superimposed on a rising then decreasing baseline of elevated $[Ca^{2+}]_{cyt}$.[33] The oscillations are not dependent on transient changes in membrane potential nor are they induced by ionomycin[33] or thapsigargin.[38] In both RBL-2H3 cells and BMMC, the oscillations are preceded by a Ca^{2+} wave that often originates in cell protrusions.[50] Ca^{2+}-entry is required to sustain oscillations beyond the initial few oscillations.[33] Localized application of immobilized antigen results in nearby repetitive Ca^{2+} puffs that are generated by Ca^{2+} release from stores without stimulating Ca^{2+} entry or formation of propagating waves.[50] These results, in the context of studies in other types of cells,[207,208] suggest that initial Ca^{2+} puffs result from IP_3-induced release of Ca^{2+} from ER which then evolve into waves and regenerative oscillations on widespread replenishment of ER stores by SOCE.

The future challenge is to define if and how multifunctional cells, such as mast cells,[209,210] encode calcium signals to promote a particular response or subset of responses. We have already noted that mast cell degranulation[14], formation of the eicosanoid precursor arachidonic acid,[15,211] chemotaxis,[11] and production of at least some cytokines[212-214] are dependent on SOCE. Yet under certain conditions mast cells can be stimulated to undergo chemotaxis[11,215,216] or produce cytokines[4] without degranulation. Preferential release of either histamine or eicosanoids has been noted with different stimulants[80,217,218] and

attributed to differences in the amplitude and duration of the calcium signal.[218] Although not specifically documented, mast cell homing and proliferation are presumably dependent calcium signals, as is true for other types of cells. The explanation as to how mast cells preferentially release mediators or undergo chemotaxis without releasing inflammatory mediators may lie in part on the nature of costimulatory signals but responses that require extensive cell remodeling such as degranulation or chemotaxis may still require calcium signals with defined spatial configurations.

Examples are insufficient to determine whether such is the case. What is known is that transient Ca^{2+} oscillations generated by release of Ca^{2+} from intracellular stores without influx are unable to support degranulation in antigen-stimulated RBL-2H3 cells.[219] However, sustained Ca^{2+} oscillations or global increases of $[Ca^{2+}]_{cyt}$ supported by influx of external Ca^{2+} are required for degranulation.[13,15,220] A similar tight dependency on SOCE was noted for the recruitment of protein kinase C and components of the ERK/PLA_2/5-lipoxygenase pathway in RBL cells activated with thapsigargin[15] or receptor agonists.[220] Stimulants such as agonists of the adenosine A_3[221] and purinergic P2Y[220] receptors elicit transient production of IP_3 and release of stored Ca^{2+} without activating sustained influx or degranulation. A confounding observation is that Ca^{2+}-entry and activation of I_{CRAC} is an all-or-none reaction in individual cells regardless of concentration of stimulant.[220] The authors conclude that the typical concentration-response curves for cell populations may reflect an increasing proportion of responding cells rather than variation in strength of signal within individual cells.

PHARMACOLOGICAL PROBES: SOCE INHIBITORS EMERGE AS POTENTIAL THERAPEUTIC AGENTS

Pharmacological probes that have been traditionally used to investigate calcium signaling include IP_3R activators (adenophostins) and inhibitors (heparin and xestospongins), SERCA inhibitors (thapsigargin and cyclopiazonic acid) and SOCE inhibitors (2-aminoethoxydiphenyl borate (2-APB)) although none of these have therapeutic application. Recently SOCE or CRAC channel inhibitors have emerged as potential therapeutic agents in the treatment of allergic and other inflammatory immune diseases.[222] The traditional inhibitors identified above were discussed in depth in a recent review[1] and they are discussed here only so far as their actions pertain to mast cells. The CRAC/SOCE inhibitors are discussed in more detail because of their therapeutic potential.

Traditional Probes for Investigation pf IP_3R, SERCA and SOCE Channels

The adenophostins (A and B) and their synthetic derivatives[223] are the most potent IP_3R agonists described to date but, because they are cell impermeant and diffuse slowly in cytosol, their administration require patch-clamp techniques. Adenophostin A has almost a hundred-fold greater affinity for IP_3R than IP_3[224] and induces quantal release of Ca^{2+},[225] intracellular Ca^{2+} oscillations and activation of I_{CRAC}.[226] However, these actions are restricted to regions where adenophostin remains localized to suggest that Ca^{2+} oscillations and entry do not extend into regions beyond the range of activated IP_3Rs.[227] In RBL1 cells, adenophostin A can induce Ca^{2+} oscillations when Ca^{2+} entry into the cell is blocked by voltage clamp to indicate that the oscillations are regenerative that is, reuptake of Ca^{2+} into ER replenishes stores sufficiently for another cycle of release and

reuptake.[226] Activation of I_{CRAC} by adenophostin A, as is true for thapsigargin or IP_3, is substantially diminished by mitochondrial depolarization.[199]

There are no ideal inhibitors of IP_3R-mediated Ca^{2+} release. The membrane-permeant macrocyclic xestospongins (B, C and D) from sponge inhibit IP_3-elicited Ca^{2+}-release without affecting IP_3 binding[228] but they have slow action and may inhibit SERCA in some types of cells.[229,230] In RBL-2H3 cells, xestospongin C (3–10 μM) inhibits intracellular release and influx of Ca^{2+}, depletion of intracellular stores by IP_3 and degranulation.[231] However, activation of SOCE after store-depletion with thapsigargin is unaffected.

The widely used SOCE activator, thapsigargin, was shown to be a mast cell activator[232-234] before it was found to deplete ER Ca^{2+}-stores by blocking SERCA-mediated reuptake of Ca^{2+} into these stores.[47] In the absence of external Ca^{2+}, thapsigargin induces a slow increase and then decrease in $[Ca^{2+}]_{cyt}$ as stores become depleted. As is the case with antigen-stimulated Ca^{2+}-deprived cells (Fig. 2), subsequent provision of Sr^{2+} or Ca^{2+} causes sustained influx of these ions in RBL-2H3 cells and BMMC. Use of this protocol and siRNA technology demonstrated that TRPC5 conducted both ions whereas Orai1 conducted only Ca^{2+} and that both channel proteins where activated by STIM1 following stimulation with either antigen or thapsigargin.[49] Thapsigargin inhibits SERCA in a stoichiometric manner by tightly binding to a single site that is common to the SERCAs but not PMCAs.[43,235] Thapsigargin has very high affinity for all three SERCA isoforms[236] such that its action is virtually irreversible.[43,237] The mycotoxin cyclopiazonic acid, in contrast, is a reversible and a less potent inhibitor of SERCA but, like thapsigargin, it suppresses SERCA Ca^{2+}-ATPase activity. The net effects of thapsigargin and cyclopiazonic acid on RBL-2H3 cells and BMMC are similar in that both activate a SOCE pathway that is permeable to Ca^{2+} and other divalent metal ions[38,48] which is impaired by microtubular disrupting agents.[238] This impairment was attributed to the inability of ER to communicate with SOCE channels in the plasma membrane or, reinterpreted in the light of current knowledge, to disrupted communication between STIM1 in ER and Orai or TRPCs in the plasma membrane.

2-APB was first reported to inhibit IP_3-induced Ca^{2+}-release from stores without affecting IP_3 binding but later studies indicated that it blocked IP_3R-mediated SOCE[239] as well.[1,240] 2-APB has a biphasic action in RBL1 cells and other types of cells.[241] I_{CRAC} is enhanced and then suppressed at, respectively, low (1-5 μM) and high (10 μM or greater) concentrations of 2-APB. Similar biphasic actions have been noted with 2-APB on overexpressed Orai proteins.[242-245] 2-APB also suppresses uptake and "leakage" of Ca^{2+} from ER pools and release from mitochondria but the compound can be a useful reagent "when used cautiously".[246]

Novel Inhibitors of CRAC and SOCE Channels

The importance of SOCE in regulating the immunological responses of mast cells, lymphocytes and other inflammatory cells has encouraged the development of selective inhibitors of SOCE as potential therapeutic agents for the treatment of allergic and autoimmune diseases. Much of the research has been conducted by the pharmaceutical industry using high-throughput screening procedures. One approach is based on fluorescence-based measurements of $[Ca^{2+}]_{cyt}$ following depletion of intracellular stores by receptor agonists linked to IP_3 production, thapsigargin, or cyclopiazonic acid. Another is based on whole cell patch-clamp technology which permits more precise characterization of SOCE inhibitors than fluorescent methods in that it can distinguish

between I_{CRAC} (i.e.,Orai) and TRPC channel activity and can exclude the possibility that the inhibitors block SOCE activity by inducing membrane depolarization.[222] These distinctions have not always been recognized and the term "CRAC inhibitor" should be used exclusively for inhibitors identified as such on the basis of electrophysiological characteristics and the term "SOCE inhibitor" when this is not the case. Either type of inhibitor, however, should be equally interesting in terms of therapeutic potential (for an informative overview see ref. 222).

More selective compounds than 2-APB were discovered after a systematic investigation of 6,5-heterocyclic compounds by investigators at the Abbott and Astellas Pharma (formerly Yamanouchi Pharmaceuticals) laboratories. Ultimately a series of highly potent 3,5-bis(trifluoromethyl)pyrazole derivatives were developed that suppressed I_{CRAC}. One such compound, BTP2 (otherwise known as YM58483 and now available commercially), selectively blocked thapsigargin-induced SOCE in Jurkat T-lymphocytes (IC_{50} = 150 nM).[247] as well as IP_3-evoked I_{CRAC} in Jurkat and RBL-2H3 cells (IC_{50} = 2.2 and 0.5 μM, respectively).[180] Interestingly, BTP2 also activates the membrane-depolarizing channel, TRPM4, at nanomolar concentrations and this action probably accounts for the potent BTP2 inhibitory effects on SOCE (as opposed to I_{CRAC}) and the associated production of IL-2 in Jurkat cells.[180] Unfortunately, similar comparisons were not reported for RBL-2H3 cells as BTP2 would appear to be useful in distinguishing between the contributions of CRAC and TRPM4 channels on SOCE and functional responses in mast cells. BTP2 also inhibits store-dependent Ca^{2+} and Sr^{2+} influx through expressed TRPC3 and TRPC5.[248] Structurally similar inhibitors of SOCE and of IL-2 production in T cells have been reported by Boehringer Ingelheim in a series of patent applications.(see ref. 222 for patent citations). BTP2 has proved effective in such models as mouse contact hypersensitivity,[247] antigen-induced eosinophilia in rodent asthma models, mouse graft versus host disease[249,250] and sheep red blood cell-induced delayed hypersensitivity in mouse. However, little is reported about its side-effects.

Other CRAC channel inhibitors have emerged from studies at Synta Pharmaceuticals. Of these, Synta 66 (GSK1349571A) was reported to be a relatively potent (IC_{50} = 1.4 μM) inhibitor of I_{CRAC} in RBL-2H3 cells and thapsigargin-induced Ca^{2+} influx (i.e.,SOCE) in Jurkat T cells without affecting TRPM4 and other channel functions.[222] Synta 66 also caused a modest reduction of T-cell proliferation and concentration-dependent reductions in expression of T-bet, IL-2 and IL-17 in vitro in biopsy specimens from humans with inflammatory bowel diseases.[251]

Although, the published data are too sparse to evaluate the efficacy and specificity of the CRAC inhibitors, the number of patent applications indicate considerable interest in CRAC inhibitors for the treatment of inflammatory diseases.[222] The identification of potent, selective inhibitors of these ion channels should be facilitated by the recent discovery of ORAI1 and STIM1 and new high-throughput ion channel inhibition assays.[252] However, the current emphasis on CRAC/Orai inhibitors should not exclude TRPCs and TRPM4 as potential targets for reasons discussed throughout this chapter. Therefore, both fluorescence and electrophysiological approaches should be pursued. Certainly, the current search for new channel inhibitors might provide new opportunities for the treatment of mast cell-related diseases. Their inherent advantage would be their rapid action because of the immediacy of the calcium signal during the activation of mast cells. Nevertheless, undesired effects will always remain a paramount issue because of the ubiquity of SOCE in cell signaling and here targeted drug disposition would be crucial in developing effective drug therapy.

DISEASE CORRELATES

No mast cell-related disease has been directly linked to genetically-related defects in calcium signaling but other human diseases have been so linked. In addition to the presenilin mutations associated with Alzheimer's disease, other examples include loss-of function mutations of Orai1[18,253] and STIM1[254] in SCID, inactivating mutations of SERCA1in Brody's disease of muscle,[182] gain-of-function mutation of SERCA2 in Darier's skin disease,[255] an inactivating mutation in Golgi SPCA1 in Hailey-Hailey skin disease,[203] and spontaneous deafness-inducing PMCA mutations.[182] While none of these mutations have been directly linked to atopic disease in humans, it has been inferred that the defects in Orai1 and STIM1 may lead to other immunodeficiency and autoimmune diseases[254] and be expected to ameliorate allergic disease on the basis of studies in experimental animal models.[127,128]

In addition to the above, gain-of-function mutations in *TRPM4* and *TRPC6* results in familial heart block type[256] and glomerulosclerosis,[257,258] respectively. As noted previously, TRPM4 is implicated in the regulation of SOCE, degranulation and migration of mast cells[178,179] but the impact of mutant TRPM4 on mast cell function has not been determined. TRPC6 is expressed in mast cells[49] but its function there is unknown. Numerous diseases have been mapped to chromosomal loci containing TRP channel genes but await demonstration of direct linkages.[259,260] Given the variety of genetically-related defects in calcium signaling in humans and the central role of calcium signaling in the activation of mast cells and other cells involved in inflammatory immune diseases, it seems a worthwhile to pursue the possibility that such defects may underlie allergic inflammatory disease in some subsets of patients.

CONCLUSION

Primary and cultured mast cell lines have been widely used for studies of calcium signaling over the past 40 years and we have learnt much about SOCE-dependent signaling from studies of these cells. Indeed, the mast cell will remain an ideal model for future research if mechanisms exist for encoding calcium signals for specific cell responses. If so, we need to understand how the activities of the various Ca^{2+} channels and sensors in different organelles are orchestrated to produce the appropriate calcium signal. Evidence points to the formation of signaling complexes embedded in specialized membrane-microdomains that facilitate the migration and interactions of channels and sensors at ER/PM and ER/mitochondrial junctions. Also unknown is whether additional flexibility in signaling is provided by the ability of Orai and TRPC isoforms to form various hetero-tetrameric combinations. These are generic questions applicable to many types of cells. There has been surprisingly little activity in regard to defects in calcium signaling in atopic and autoimmune diseases even though there are now well documented examples of genetic diseases related to defects in the operation of SOCE, SERCA, TRPM4 and NCX. We suspect that this topic as well as examination of the novel SOCE/CRAC inhibitors will be key topics for future research on mast-cell related diseases.

ADDENDUM

Since completion of this manuscript Srikanth et al (Nature Cell Biol 2010; 12:436-46) have described a CRAC regulator (CRACR2A) that might account for the suppression of CRAC activity by high $[Ca^{2+}]_{cyt}$. At basal $[Ca^{2+}]_{cyt}$ CRACR2A enhances binding of STIM to Orai thereby promoting SOCE but it dissociates from the STIM/Orai complex at high $[Ca^{2+}]_{cyt}$ and inhibits SOCE. Apparently, CRACR2A and calmodulin compete for the same Orai1 binding site which is consistent with the previously described inactivation of Orai1 by calmodulin.

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CHAPTER 6

SPATIO-TEMPORAL SIGNALING IN MAST CELLS

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Abstract: This chapter summarizes the evidence for localized signaling domains in mast cells and basophils, with a particular focus on the high affinity IgE receptor, FcεRI and its crosstalk with other membrane proteins. It is noteworthy that a literature spanning 30 years established the FcεRI as a model receptor for studying activation-induced changes in receptor diffusion and lipid raft association. Now a combination of high resolution microscopy methods, including immunoelectron microscopy and sophisticated fluorescence-based techniques, provide new insight into the nanoscale spatial and temporal aspects of receptor topography on the mast cell plasma membrane. Physical crosslinking of FcεRI with multivalent ligands leads to formation of IgE receptor clusters, termed "signaling patches," that recruit downstream signaling molecules. However, classes of receptors that engage solely with monovalent ligands can also form distinctive signaling patches. The dynamic relationships between receptor diffusion, aggregation state, clustering, signal initiation and signal strength are discussed in the context of these recent findings.

INTRODUCTION

Engagement of the high affinity IgE receptor, FcεRI, is the principal physiological stimulus for mast cell degranulation. This tetrameric receptor is composed of the IgE-binding α subunit, two γ subunits and a tetraspan β subunit. There are a total of three ITAMs (immunoreceptor tyrosine-based activation motifs), one in the carboxy terminus of the β subunit and one in each of the paired γ subunits. Activation occurs when IgE binds to polyvalent antigens, crosslinking a minimum of two FcεRI-IgE complexes thereby initiating a tyrosine kinase cascade that triggers histamine release and de novo synthesis of leukotrienes, prostaglandins[1] and an impressive list of cytokines and chemokines.[2]

Mast Cell Biology: Contemporary and Emerging Topics, edited by Alasdair M. Gilfillan and Dean D. Metcalfe.

Biochemical details of this cascade have been the subject of many reviews, including Gilfillan and Rivera[3] and other chapters in this volume.

In the 25 yr period spanning from 1975-2000, the FcεRI was the subject of pioneering studies that measured the mobility and behavior of membrane proteins. Emerging biophysical techniques, such as Fluorescence Recovery After Photobleaching (FRAP), postfield relaxation after in situ electromigration and time-resolved phosphorescence anisotropy, were used to arrive at estimates of lateral diffusion rates of 1.5-4 × 10^{-10} cm^2/s for resting receptors.[4,5] Rotational correlation values ranged from 23-65 ms[6-8] with slower values attributable to measurements at low temperatures. In these early studies, incomplete recovery from photobleaching was already recognized as an indication that a small fraction of receptors were immobile in resting cells, with marked increases in the immobile fraction after antibody-mediated crosslinking and the concomitant observation of microaggregates and patches visible in the fluorescence microscope.[4,9] Rotational mobility was also markedly restricted after receptor crosslinking.[6] These and related studies led to a number of important hypotheses that are still being vigorously tested today. These include revision of classical Brownian diffusion models of cell membrane proteins to explain restricted lateral mobility[10] and the widely-held concept that FcεRI immobilization must be necessary for signal initiation.[11,12] Antigen valency and structure, particularly as it relates to the orientation and distance of FcεRI subunits within aggregates, also continues to be a highly relevant topic[13] that is strongly linked to both receptor immobilization and signaling efficiency.

MICRODOMAINS, RAFTS AND ISLANDS

For almost forty years, the fluid mosaic model has provided a conceptual framework for plasma membrane structure.[14] This model, which integrated many studies from the preceding decade, viewed cellular membranes as two-dimensional solutions composed of integral membrane proteins within a lipid matrix. It proposed that most membrane constituents diffuse rapidly and are randomly arranged, but it acknowledged the possibility of ordered membrane regions based on early EM images of clustered proteins. Variations of this model arose however, beginning with the terms "lipid rafts" and "liquid ordered and disordered domains".[15-17] Seminal studies by several groups working in the mast cell field provided compelling evidence for the lipid raft hypothesis, based in large part upon the recovery of FcεRI and its signaling partners or regulators in light fractions of sucrose gradients after stimulation.[18-21] While the popularity of the lipid raft concept energized the membrane biology community, artifacts associated with merging of compartments by detergent treatment may have also led to misconceptions. This is now the prevailing view in the field, as evidenced by recent reviews stressing the heterogeneity of the membrane and the likelihood that microdomains are both small, readily exchangeable and complex.[22-25] Clearly, more sophisticated and direct measurements are needed to both refine and correct this model.

An alternative view has re-emerged for membrane organization, referred to as the "protein islands" model.[26,27] It is similar to the predictions of Yechiel and Edidin,[28] who proposed that "plasma membranes are organized into protein-rich lipid domains, separated by a protein-poor lipid continuum." Importantly, the protein islands model explains membrane heterogeneity, based upon the strong segregation of distinct protein subpopulations with islands and acknowledges rich association with cholesterol and cytoskeletal elements. The model revives appreciation for the influence of local protein density on diffusivity,[29] a concept that is reinforced by evidence that protein-rich confinement zones transiently trap diffusing membrane proteins on T cells.[30]

ELECTRON MICROSCOPY PROVIDES A BIRD'S EYE VIEW OF FcεRI SIGNALING PATCHES

Scanning Electron Microscopy (SEM) provided the first nanoscale resolution images of immuno-gold labeled FcεRI "patches",[31] that were hinted at in the early immunofluorescence experiments.[4] The size of patches and the time course for their formation, was shown by SEM to be dependent on antigen dose.[32] By 2000, our group had developed the technology to prepare native membrane sheets ripped from rat basophilic leukemia (RBL-2H3) cells at various stages of activation.[33] With the improved resolution of Transmission Electron Microscopy (TEM), these preparations could be labeled more efficiently by smaller nanogold particles and by other novel nanoparticles such as quantum dots.[34] The sheets also permitted access to the cytoplasmic face of the membrane for labeling intracellular proteins recruited during signaling.

Figure 1 provides typical views of FcεRI signaling patches before (1A) and after 2 min stimulation with antigens with different valency (1B,C). Cells in Figure 1B were

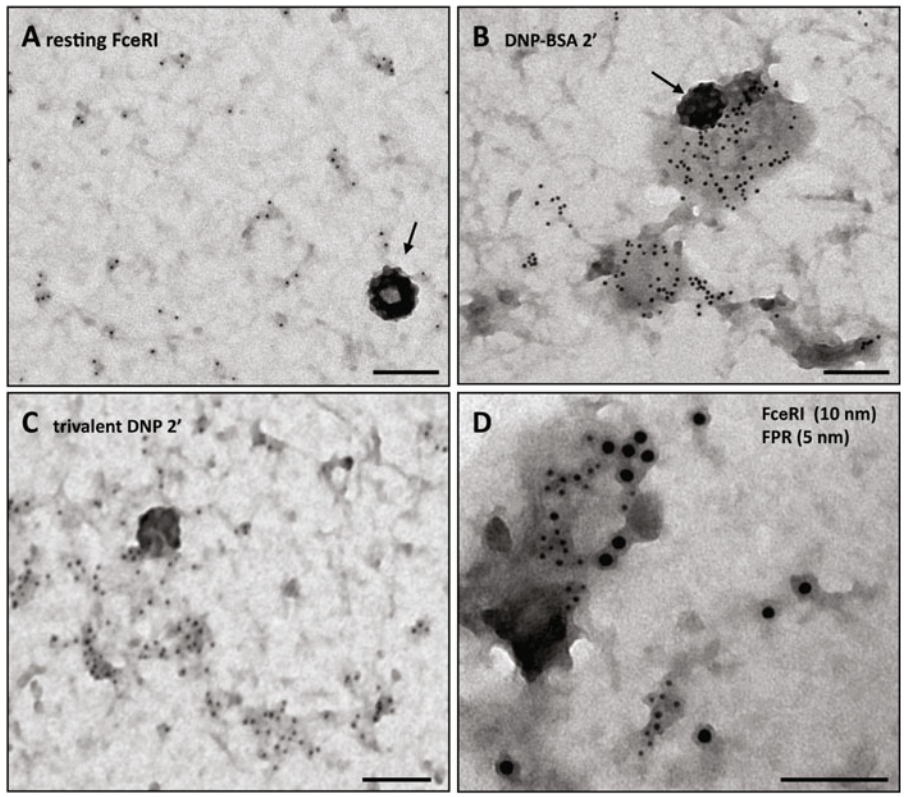

Figure 1. Electron microscopy reveals the spatial distribution of membrane receptors. A) RBL membrane sheets labeled with immunogold as in reference 33 to show that resting FcεRI are found as singlets and in small clusters. Arrow marks a clathrin-coated pit. B) Two min after stimulation with 0.1 μg/ml DNP_{24}-BSA, larger clusters are seen to form. C) Cells stimulated with a PEG-based flexible trivalent DNP ligand (10 nM) also demonstrate extensive FcεRI aggregation. D) Simultaneous activation FcεRI (10 nm gold) and FPR (5 nm gold) leads to coclustering of the receptors. Scale bars = 100 nm.

activated with DNP_{24}-BSA (0.1 μg/ml) while cells in Figure 1C were stimulated with a PEG-based flexible trivalent DNP ligand (10 nM). Note that, even in the resting state, receptors are not completely random (Fig 1A). Small clusters, ranging from 2-5 receptors, are typically found in addition to singlets. FcεRI clusters generally increase in size after antigen-mediated crosslinking,[32] although the increase is somewhat smaller in response to ligands of low valency or even with low concentrations of polyvalent antigens.[35] However, comparison of secretory responses for the two antigen conditions illustrated here (60-70% for DNP_{24}-BSA versus 10-30% for the PEG-trivalent ligand) makes it clear that *cluster size does not predict signaling output*.

To further drive this point home, crosslinking with a panel of divalent antibodies against the FcεRI α subunit yielded similar, small cluster sizes.[36] Importantly, only the signaling-competent antibodies showed dissociation from Lyn and recruitment of significant amounts of Syk tyrosine kinase to these receptor clusters.[36] Together with other reports,[13] these data support the conclusion that dimerizing agents *can* activate the FcεRI, provided that crosslinking induces appropriate orientation or conformational changes in pairs of receptors.

Of note, the large signaling patches induced by multivalent antigen are often bordered by coated pits poised to internalize receptors (Fig. 1B, arrow). This observation is highly reproducible, despite the fact that depletion of clathrin fails to inhibit FcεRI endocytosis after activation (ref. 37 and our unpublished results). We speculate that, like the BCR,[38] compensatory nonclathrin mediated internalization pathways ensure that highly crosslinked FcεRI are removed from the mast cell surface.

GPCRs BOUND TO MONOVALENT LIGANDS ALSO FORM SIGNALING PATCHES

The N-formyl peptide receptor (FPR) is naturally co-expressed with the IgE receptor on human basophils. In a study of RBL-2H3 transfectants stably expressing the FPR, this GPCR was observed to form large clusters in response to addition of monovalent ligand.[39] Ligand-bound FPR cluster rapidly and recruit the heterotrimeric G-protein, Gi. FPR are slowly endocytosed and, by 5 min, most clusters remaining on the surface contain arrestin and very little Gi. We also found that, when ligands for both FPR and FcεRI were added simultaneously, there was a marked increase in colocalization of the two receptor clusters (Fig. 1E). This study suggested that crosstalk between the tyrosine kinase pathway of the FcεRI and the G-protein-coupled pathway of the FPR occurs locally. Barisas and colleagues have proposed that crosstalk between FcεRI and the inhibitory receptor, MAFA, may also occur in rafts or specialized domains.[40] Together with evidence for clustering of growth factor receptors.[41,42] These data also support the notion that receptor clustering is not solely a property of physical crosslinking by multivalent ligand (as is the case for FcεRI) but a more generalized architecture for receptors during active signaling.

SIGNALING PATCHES AS SPECIAL FEATURES OF THE PROTEIN ISLAND NETWORK

Experiments comparing the distributions of *all proteins* in T-cell and RBL membranes shed some light on the issue of colocalizing receptors.[26] When bulk

membrane proteins were labeled for electron microscopy with probes targeted at SH-groups, the gold label concentrated in dark regions of membrane that also label for cholesterol. Furthermore, nonraft and raft markers were constructed by expressing tagged versions of N-terminus of Lck with and without mutation of the palmitoylation sites. Statistical analysis using the Ripley's K function confirmed that the two markers were strongly segregated. These results led us to propose a new model for membrane organization, where proteins organize into complex, cholesterol-rich "islands" in a largely lipid sea. This hypothesis was recently supported by the sophisticated PALM technique (photoactivation localization microscopy).[27] We speculate that signaling patches represent specialized domains within islands that initiate, amplify and control signal propagation in a spatially restricted manner.

SINGLE PARTICLE TRACKING ALLOWS DIRECT OBSERVATION OF DIFFUSING FcεRI

We have recently developed a method to directly observe the diffusive behavior of individual FcεRI, before and after crosslinking with ligands of different valency. As described in our recent publications,[34,35] monovalent Quantum Dot (QD)-labeled IgE can be reliably prepared and used to "prime" FcεRI at sparse densities that allow for identification and tracking of individual QD-IgE-receptor complexes diffusing on the surface of live cells. The use of QDs provides several advantages. Their high brightness and photostability allows for long-term single molecule imaging. QDs have broad excitation, yet narrow emission, spectra allowing for simultaneous excitation and detection of spectrally distinct QDs using a single excitation wavelength and filter-based detection. The use of two- or four-color emission beam splitters and sensitive emCCD cameras permits fast and well-resolved collection of fluorescence from 2-4 spectrally distinct probes simultaneously at the single QD level.

Using single particle tracking, we have characterized the diffusive behavior of FcεRI (Fig. 2A), which ranges from highly mobile to confined to immobile. Temperature plays a significant role, with ~2 fold faster diffusion and larger corral sizes measured at physiological temperature (35-37°C) as compared to room temperature.[34] Even in the resting state, a small fraction of receptors are found to be at least transiently immobile (classified by a diffusion coefficient <0.001 $\mu m^2/s$). Individual receptors can switch rapidly and apparently randomly between these diffusion states (see movies in ref [34]).

The ability to distinguish two or more QD colors was a critical factor in determining whether resting FcεRI, observed by EM to be distributed in small clusters, interact with each other sufficiently strongly to drive the clustering. The best evidence for this possibility would be the detection of "correlated motion" over a significant period of time. As shown in Figure 2C-D, we often observe QD-labeled IgE receptors in close proximity. Figure 2C illustrates a case of two receptors which remain close (<500 nm) for several seconds. However, the plot at the right shows that their movements remain uncorrelated. In Figure 2D, two receptors move close to each other for a short time then separate, moving over 2 microns apart before reversing directions and crossing paths again. Analysis of >1,000 close-approach events imaged at 100 frames/sec revealed that correlated motion was not occurring, indicating that the resting receptors do not form a stable complex.[34] Thus, the most obvious interpretation is that resting FcεRI can briefly share residency in the same microdomain, but that their associations (if any) during this

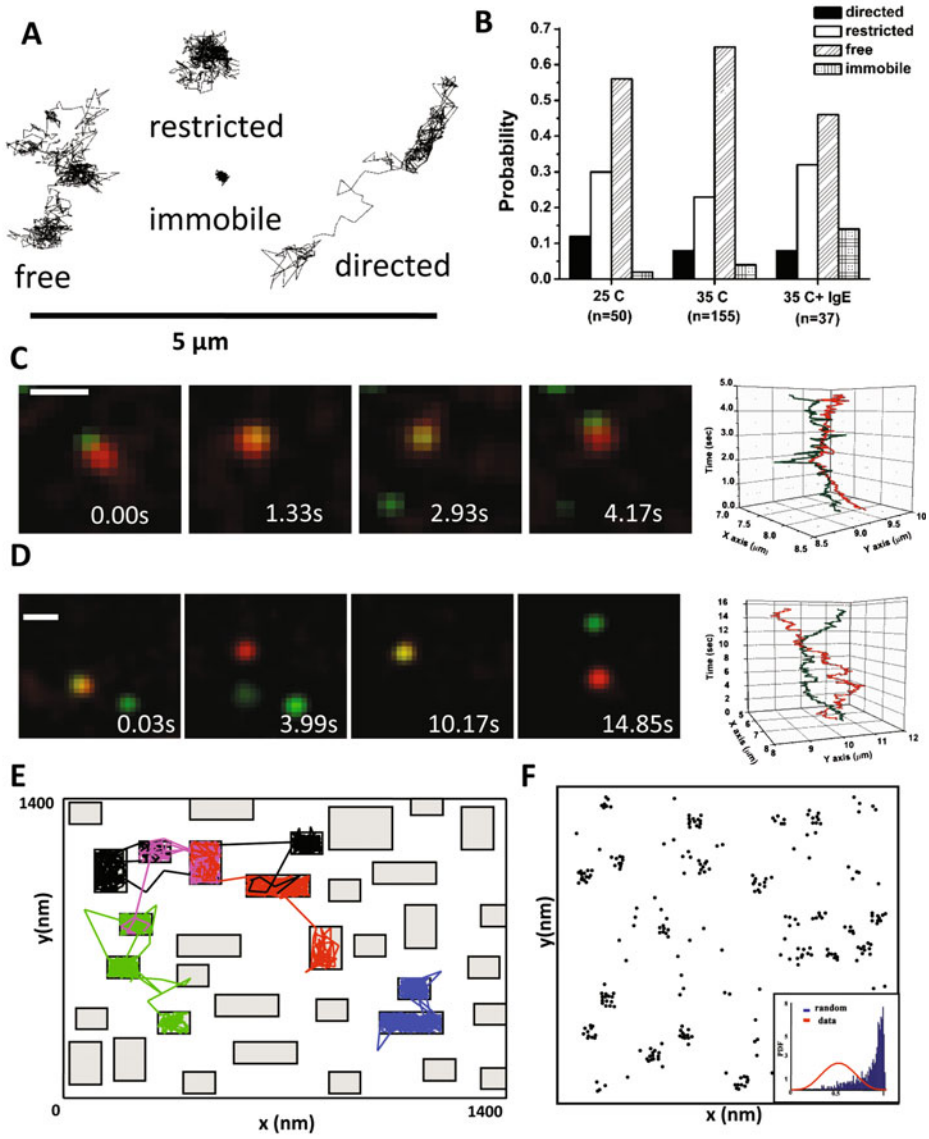

Figure 2. Characterizing FcεRI diffusion. A) Example trajectories of individual FcεRI motion as determined by single quantum dot tracking. The expected four modes of motion are observed: free, restricted, directed and immobile (see Andrews et al, 2008). B) Distribution of the modes of motion under different conditions. C,D) Two-color imaging allows us to simultaneously track receptors labeled with QD655-IgE and QD585-IgE. Note that receptors often maintain close proximity but do not demonstrate correlated motion, indicating that the close proximity is due to coconfinement in a microdomain, not receptor interactions. (C,D from reference 34) E) Example simulation from the Constrained Brownian Motion algorithm. Proteins in the simulation are considered as individual particles or agents that move at each time step (see ref. 43). The membrane can be further subdivided into domains that are lipid-rich (fast diffusion for proteins, white space) and protein-rich (2-fold slower diffusion for proteins in the simulation, grey regions). Slower movement of receptors through the protein-rich domains leads to clustering of resting receptors (F) similar to that seen in electron microscopy images (Fig. 1).

residency period are very weak. This is consistent with time-resolved phosphorescence polarization studies, which concluded that the rotational mobility of resting receptors is neither hindered by direct association with the cytoskeleton or by aggregation.[6]

MODELING THE INS AND OUTS OF RESTING RECEPTOR CLUSTERS

We have used stochastic modeling approaches to test the possibility that transient residency of resting receptors in islands or rafts provides a plausible explanation for their nonrandom distribution. For these simulations, the 2-D membrane is represented by a Cartesian plane with a periodic boundary such that receptors that leave the plane re-enter on the opposite side of the membrane. Species in the simulation are considered as individual particles or agents that move at each time step under a Constrained Brownian Motion algorithm.[43] The membrane can be further subdivided into domains that are lipid-rich (fast diffusion for proteins) and protein-rich (2-fold slower diffusion for proteins in the simulation) (Fig. 2E). Although we first applied this technique to the EGFR, the approach is applicable to virtually any resting membrane receptor (including FcεRI) provided diffusion rates are experimentally defined. Even if membrane proteins begin the simulation at random locations, a pattern of clustering will form that closely matches that observed by immunoelectron microscopy and similarly passes the Hopkins test (Fig. 2F). We conclude that, even when receptors are in constant motion, short duration trapping in protein-rich microdomains (islands) can result in clustering.

Consistent with this hypothesis is prior evidence that FcεRI diffusion is profoundly influenced by protein crowding. Thomas et al[29] found that FcεRI slowed significantly within densely populated poles that developed on RBL cells exposed to electric fields. In that study, significant effects were revealed only on membranes of osmotically shocked RBL cells, which released constraints of the cortical cytoskeleton. Other experiments during the early 1990s demonstrated that crowding slows diffusion of membrane receptors in artificial bilayers.[44,45] Of course, an intact cytoskeleton also exerts a strong influence on receptor diffusion, by serving as physical barriers or corrals[34] and by anchoring protein islands.[26] The cytoskeleton will be further discussed in sections below.

IMMOBILIZATION OF CROSSLINKED FcεRI IS DEPENDENT ON DOSE AND ANTIGEN VALENCY

Tracking of QD-IgE bound receptors permitted us, for the first time, to observe the transition from resting to crosslinked receptors in real time (Fig. 3A). As predicted, based upon prior reports of FcεRI immobilization after crosslinking,[4,11,46] we observed that stimulation with modest to high doses of highly polyvalent antigen (DNP_{24}-BSA, 0.1-10 μg/ml) resulted in rapid immobilization (Fig. 3A). Our real-time assay was directly able to determine the kinetics of this change in mobility, found to occur in less than 20 s. Somewhat unexpectedly, we found that low doses of the same highly multivalent antigen failed to induce immobilization despite robust stimulation of degranulation.[35] We similarly found that even high doses of low valency antigens (DNP_2-BSA, DNP_4-BSA), also shown to induce degranulation, did not cause receptor immobilization (Fig. 3B).

These results firmly lead to the conclusion that immobilization is not a requisite event for *signal initiation* from the crosslinked FcεRI. However, it is intriguing to consider this

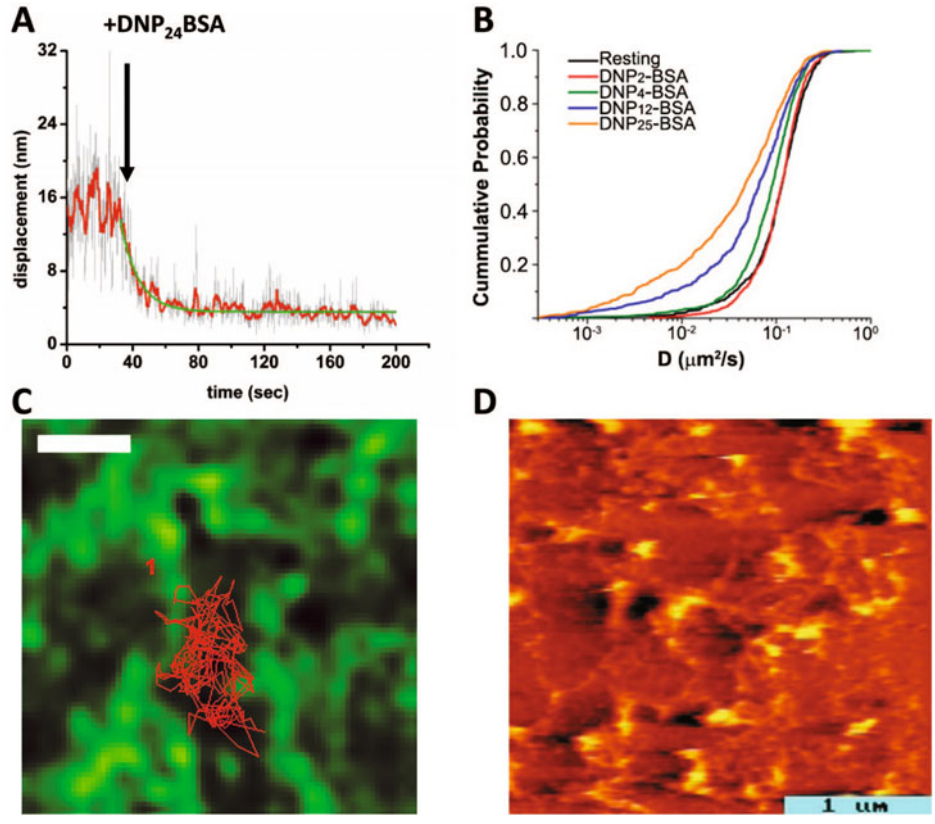

Figure 3. A) Plot of the average displacement of QD-IgE-FcεRI complexes between subsequent frames over the time course of a single movie. After addition of 1 μg/ml DNP_{24}-BSA, the displacement is seen to rapidly decrease, indicating a slow-down in receptor mobility (see ref. 34). Red line is 10 frame averaging. Green line is exponential fit to the decay. B) Cumulative Probability Analysis plot comparing the diffusion coefficients (D) of QD-IgE-FcεRI before and after crosslinking with antigens of different valency. Note that with DNP_2-BSA (red) receptor mobility did not change from resting (black) even though degranulation was seen under these conditions. Higher valencies did cause receptor slow-down, presumably due to the formation of larger aggregates. (Reproduced with premission from: Andrews NL et al. Immunity 2009; 31(3):469-479;[35] ©2009 Elsevier.) C) Tracking of QD-IgE-FcεRI on cells expressing GFP-actin (green) shows that the receptor trajectory (red line) is confined by the actin cytoskeleton (green). Here, cells were treated with PMA that stabilized the actin filaments (see ref. 34). Scale bar, 1um. D) AFM image revealing the topography of the cytoplasmic face of an RBL membrane sheet. The image is pseudo-colored to indicate heights ranging from 5-6 nm (dark red, the width of a lipid bilayer) to 30 nm (hot yellow) above the substrate. Raised domains dot the landscape and are connected by many thin filaments. (Reproduced with permission from: Frankel DJ et al. Biophys J 2006; 90(7):2404-2413;[58] ©2006 Elsevier.)

in the broader context of previous work using structurally defined ligands and dimerizing antibodies for FcεRI stimulation (reviewed in see ref. 13). There are strong correlations between *signaling potency* and the predicted oligomeric state of crosslinked receptors. Dimerizing reagents, including both divalent ligands and antibodies directed at either the FcεRI α subunit or at IgE, exhibit a wide range of stimulatory capacity. It is possible for such reagents to induce immobilization in the absence of signaling, at least as measured by

degranulation and Ca^{2+} mobilization.[47] Bivalent ligands with flexible PEG-based spacers fail to stimulate measurable secretion[48] and can even be inhibitory.[49] In general, higher oligomers induced by higher valency antigens—or further crosslinking of antibody-bound complexes—generate better signaling from the RBL-2H3 cell line over a broad dose response range. A number of factors have been implicated, including overriding constraints imposed by cyclic dimers as well as the improved geometry, spacing and orientation of receptors in large aggregates (see discussion in ref. 13). Lateral forces may also be a factor, based upon observations that liposomes or lipid bilayers bearing haptens for IgE can recruit large numbers of receptors to the adhesive surface and can induce weak but measurable secretory responses[50,51] IgE-FcεRI complexes engaged by lipid-conjugated monovalent hapten form a "synapse" and induce degranulation while remaining highly mobile, further demonstrating that immobilization is not required for signal initiation.[52]

We note that maximal secretory responses of bone marrow derived mast cells (BMMC) and human basophils occur over a much narrower range even for the highly polyvalent antigens,[35,53] often abruptly dropping off at modest concentrations of crosslinking reagents. This phenomenon has been referred to as "high dose inhibition" and may be attributed to active negative regulatory mechanisms that dominate in FcεRI-bearing primary cells. In support of this, we found that receptor immobilization can trigger internalization even in the absence of signaling.[35]

THE INFLUENCE OF THE CORTICAL CYTOSKELETON

The actin cytoskeleton has long been proposed to form "corrals" or "picket fences" capable of restricting movements of membrane proteins.[54-56] By coupling QD-IgE single particle tracking with TIRF (total internal reflection microscopy), we were able to directly test this widely-held theory.[34] To perform this series of experiments, RBL cells were selected that stably express GFP-actin. The movements of resting QD-labeled IgE receptors were then tracked relative to actin filaments at the lightly adherent surface of live cells settled on coverslips. Images were captured at a rate of 35-100 frames/second. As illustrated in Figure 3C, QD-labeled receptors were shown to be markedly restricted by the network of actin cables juxtaposed to the inner membrane. Moreover, because the actin network is dynamic, the shapes of the corrals were shown to be constantly changing and therefore frequently permit QD-labeled receptors to "slip" between openings to previously inaccessible compartments.

The GFP-actin cables, seen by TIRF, span distances of several microns. We have used high resolution microscopy techniques to capture fine details of cortical cytoskeletal elements that are too fine to resolve in the fluorescence microscope, even in TIRF mode that helps to eliminate out-of-focus light.[57,58] The image in Figure 3D shows the topographic detail of the cytoplasmic face of a hydrated membrane sheet prepared from an RBL cell. It was acquired on the AFM (Atomic Force Microscope) of our collaborator, Alan Burns, at Sandia National Laboratory. The image is pseudo-colored to indicate heights ranging from 5-6 nm (dark red, the width of a lipid bilayer) to 30 nm (hot yellow) above the substrate. Raised domains dot the landscape and are connected by many thin filaments. This image and others obtained on the transmission electron microscope (see cables in the images in Fig. 1), led us to conclude that the cortical cytoskeleton links protein islands in the RBL membrane. We found that labels for actin and myosin do not uniformly label the fine meshwork.[57] Instead actin and myosin are usually concentrated at junctions in

the network. This leads us to speculate that actin bundles are only a minor fraction of the network, providing an explanation for the fact that latrunculin treatment only modestly enhances the mobility of molecules in single particle tracking experiments.[59,34] Lillemeier and colleagues[26] found that protein islands are smaller but persistent in T-cell membranes when their cytoskeletal tethers are disrupted by latrunculin treatment. Dense cytoskeletal networks or direct associations with crosslinked FcεRI may actually limit the signaling process, as suggested by the work of Seagrave and Oliver[32] and Holowka and Baird[60] Seagrave and Oliver[61] noted that cytochalasin treatment reduced the size of FcεRI aggregates observed by SEM, leading to the hypothesis that very large aggregates signal poorly.

INTRACELLULAR PROTEIN-PROTEIN INTERACTIONS PROVIDE AN ADDITIONAL MECHANISM TO STABILIZE SIGNALING PATCHES: THE CASE OF LAT

Like other immunoreceptors, signal propagation following FcεRI crosslinking involves the recruitment of a large number of proteins that contain binding motifs, such as SH2 (src homology 2), SH3 (src homology 3), PH domains, PTB domains and others.[3,62] Large macromolecular complexes can form based on these interactions, with the potential to induce aggregation and stabilize clusters of receptors and other signaling proteins. Goldstein and colleagues have used mathematical modeling to validate intracellular cross-bridging as a mechanism for growth and stabilization of large clusters of the scaffolding protein, Linker for Activation of T cells (LAT).[63] The model is based upon the ability of Grb2 to bind via its SH2 domain to phosphorylated LAT and to two molecules of SOS through its two SH3 domains.[64,65] Thus multivalent, cooperative protein-protein interactions that propagate at the cytoplasm-membrane interface may explain, in large part, the observation that LAT clusters markedly increase in size following FcεRI crosslinking[57] or TCR activation.[27,64] It seems likely that this paradigm will be broadly applicable to other transmembrane proteins that participate in complex signaling cascades.

WHAT ABOUT LIPIDS?

The local lipid environment of immunoreceptors remains enigmatic. In 2002, Jacobson and Anderson proposed that membrane proteins might be surrounded by a lipid shell.[66] Proof for this attractive concept has eluded investigators to date, mostly because of technical limitations. However, evidence for strong lipid association with FcεRI dates back to the work of Rivnay, Metzger and colleagues.[67,68] The addition of lipids to detergent micelles delayed the slow dissociation of FcεRI subunits, with the best protection provided by dipalmitoylglycerol and cholesterol.[69]

If a lipid shell envelopes FcεRI, cholesterol is perhaps our best candidate. However, results from multiple laboratories remain complex and, in our opinion, controversial. For example, RBL cells depleted of cholesterol after mBCD treatment show profound defects in membrane topography, including widespread "flat" clathrin arrays and reduced overall height of all membrane domains.[58] These broad effects make it difficult to interpret differing reports that cholesterol depletion either inhibits the entire FcεRI tyrosine kinase cascade[70] or has little effect on FcεRI-induced phosphorylation but differential effects on calcium mobilization and degranulation.[71,72]

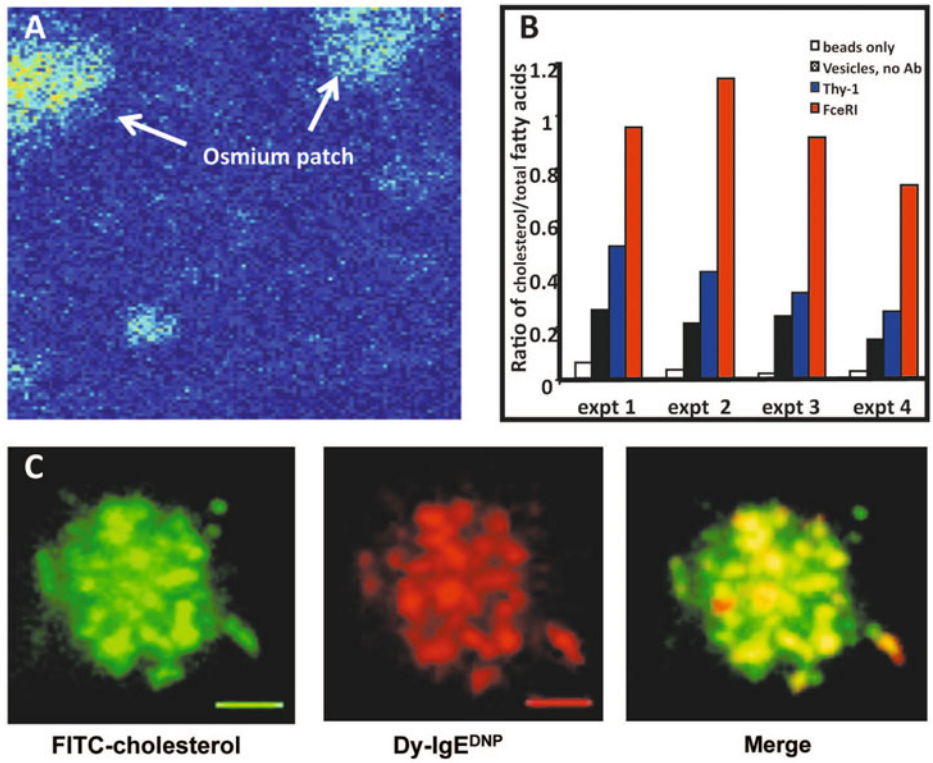

Figure 4. A) Pseudo-colored X-ray spectral electron microscopy image, where arrows point to "hot spots" for osmium staining over a region of a membrane sheet. Images were captured on a Phillips FEI Tecnai F30-ST equipped with an EDAX r-TEM SUTW energy dispersive X-ray detector. (Reproduced with permission from: Pike LJ et al. J Biol Chem 2005; 280(29):26796-26804.[75]). B) Results from lipid analysis of IgE-containing vesicles (red) compared to Thy-1-containing vesicles (blue). Lipid content in IgE-vesicles was 50% cholesterol, compared to less than 25% in Thy-1 vesicles. C) Simultaneous imaging of cholesterol-18 FITC (green) and IgE-FcεRI (red) in the membrane of an RBL cells as it contacts a lipid bilayer containing monovalent DNP. Cholesterol and FcεRI are seen to colocalize in patches that travel together during synapse formation.[52]

We have focused on alternative approaches to evaluate the lipid environment of FcεRI in resting and activated states. We previously made use of the capabilities of X-ray spectral electron microsopy,[73] where specific elements in the sample emit characteristic X-rays when excited by the electron beam and the intensity of the signal relates to its concentration. Data can be acquired in scanning mode on STEM instruments, reporting pixel-by-pixel values for specific elements at nanometer resolution; operating in TEM mode, one also can acquire a more traditional image of the sample that is based on contrast. We focused on the distribution of osmium, a contrast agent commonly used in electron microscopy due to its ability to react with double bonds in lipids. As shown in Figure 4A, this method revealed that membranes concentrate osmium in patches on the membrane; and this observation strongly suggested that lipids with double bonds (a category that includes cholesterol) were unequally distributed.

Our next approach was to isolate plasma membrane-derived vesicles for lipidomics analysis in collaboration with Robert Murphy (Univ. of Colorado), with a primary goal of avoiding detergent expected to disrupt native structure. Our strategy was to crosslink IgE receptors or GPI-anchored Thy-1 on the surface of RBL cells with specific antibodies, forming large patches enriched in either activated receptors or aggregated Thy-1.[74] Cells were rapidly chilled and mechanically disrupted to vesiculate the plasma membrane. After a series of steps to reduce contamination from unbroken cells, etc, we then used immunomagnetic bead isolation to isolate right-side-out vesicles containing IgE receptors or Thy-1. Enrichment was confirmed by 2-D gel electrophoresis (to evaluate the protein content) and then samples were prepared and sent to Denver. This process was repeated over several years for an in depth analysis of the levels of fatty acid saturation, cholesterol levels and lipid composition. This study generated several significant findings: (1) Both vesicle preparations contained a complex set of plasma membrane phospholipids, as analyzed by mass spec analysis; (2) Lipid content in vesicles immuno-isolated with anti-IgE was 50% cholesterol, compared to less than 25% in Thy-1 vesicles (Fig. 4B); (3) Less than 50% of the fatty acids in either domain were saturated, inconsistent with the concept of liquid ordered domains; and (4) IgE receptor-containing vesicles contained 2-3 times more sphingomyelin and plasmalogens than the Thy-1 domains. The plasmalogen finding is particularly intriguing, since the vinyl ether linkage at the sn-1 position introduces a double bond that is very near the lipid head group. Plasmalogens were also found to be enriched in lipid raft fractions containing the EGFR.[75] Although plasmalogens are widely distributed in nature, roles for these lipids remain poorly defined.[76] Remarkably, new mouse models with ether lipid deficiencies have severe defects, including arrest of spermatogenesis, cataracts and deficiencies in CNS myelination. These problems have been linked to impaired intracellular cholesterol distribution, plasma membrane function and ER/Golgi structural changes (reviewed in see ref. 76). On an interesting note, mast cells from cholesterol-deficient SLOS mice exhibit hyperdegranulation and constitutive cytokine production, which may be linked to down regulation of Lyn acting in its negative regulatory role.[77]

Recently we employed new strategies to allow us to track cholesterol in live cells. Working with Jeri Timlin at Sandia National Laboratories, we have tested several fluorescent cholesterol derivatives in RBL-2H3 cells. Our best success is with cholesterol-18 FITC that has a long linker for the chromophore (Fig. 4C). This probe is easily tracked when imaging in TIRF mode and forms patches that markedly travel and colocalize with FcεRI recruited to lipid bilayers presenting monovalent, mobile ligands.[53] These patches are remarkably similar to the microclusters of TCR and BCR that travel to the well described synapses of T cells[78] and B cells.[79] This was the first evidence that microclusters of immunoreceptors are enriched in cholesterol as they move to the synapse.

It remains to be determined which of cholesterol's biochemical and biophysical properties predominate in the regulation of membrane architecture: its ability to influence membrane rigidity and fluidity; its affinity for sphingolipids and disaturated phosphatidylserine;[80] its ability to bind directly to specific proteins;[81,82] or its importance for membrane curvature.[83] For the FcεRI, we are struck by the enigma of its association with mobile, but not immobile, receptors at the immune synapse.[52] If cholesterol comprises part of the lipid shell around FcεRI, it must be sufficiently weak that dissociation occurs if receptors become trapped by binding to an immobile ligand. Since receptors trapped in this way cause massive degranulation, it also appears that cholesterol is not an absolute requirement for signal transduction to commence.

CONCLUSION AND FUTURE DIRECTIONS

As the entry point for signaling input, the plasma membrane continues to be the focus of intense study. Its constituents occupy a surprisingly complex 2-D environment, where interactions are dependent on encounters in confined and constantly rearranging spaces. Perhaps the greatest next challenge is to capture the subsecond temporal details of signal transducers recruited to FcεRI oligomers stimulated with defined ligands, as well as to downstream signaling scaffolds such as LAT. This will be an important goal, since population readouts such as phosphorylation patterns of FcεRIβ, Syk and LAT fail to correlate well with downstream responses such as degranulation.[84]

We anticipate the development of such measurements in the near future, since innovation has been a hallmark of the mast cell imaging community, often leading the way for the entire field of receptor biology. Examples include the development of GFP/SH2 and GFP-PH domain fusion proteins as reporters of protein translocation to the plasma membrane probes in response to FcεRI activation.[85] Fluorescence correlation spectroscopy (FCS) has been creatively applied by the collaborative team of Webb, Baird and Holowka[86] to measure association between FcεRI and Lyn tyrosine kinase on live cells following antigen stimulation. This important subject deserves revisiting, since the authors' choice of room temperature measurements (to slow internalization and membrane ruffling) likely also delayed the apparent time course of Lyn recruitment (5-6 min after antigen addition). Our group has developed multi-color quantum dot probes to directly observe the formation of FcεRI aggregates, to track the correlated motion of small aggregates and to observe the abrupt immobilization of larger aggregates.[34,35] FCS and SPT represent powerful technologies for measuring the dynamics of protein-protein interactions. In addition, emerging super-resolution and correlation imaging technologies hold much promise for the quantitative measurement of protein-protein interactions in living cells (reviewed in see ref. 87). Such advances in imaging techniques will allow us to take the next step—beyond the plasma membrane—to look at the cytoplasmic protein dynamics and interactions that propagate the message initiated by FcεRI aggregation.

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CHAPTER 7

THE MECHANISMS OF EXOCYTOSIS IN MAST CELLS

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Abstract: Upon activation through high affinity IgE receptors (FcεRI), mast cells (MCs) can release up to 100% of their content of preformed mediators stored in cytoplasmic secretory granules by compound exocytosis. This causes Type I immediate hypersensitivity reactions and, in the case of inappropriate activation by allergens, the symptoms of allergy. Recent work has uncovered a central role of SNARE (Soluble *N*-ethylmaleimide-Sensitive Factor (NSF) Attachment Protein (SNAP) Receptors) proteins in regulating the numerous membrane fusion events during exocytosis. This has defined a series of new molecular actors in MC exocytosis that participate in the regulation of membrane fusion and the connection of the fusion machinery with early signaling events. The purpose of this chapter is to describe these proteins and provide a brief overview on their mechanism of action.

INTRODUCTION

MCs are specialized secretory cells that respond to inflammatory signals with the massive secretion of a wide range of inflammatory products. Some, like histamine, proteases and proteoglycans, are stored in cytoplasmic secretory granules (SGs) and can be released by exocytosis upon stimulation, ensuring an immediate and maximal biologic effect. They also newly synthesize arachidonic acid metabolites (LTC4, PGD2), as well as various inflammatory cytokines/chemokines released by vesicular transport.[1] Together these account for the biological activities of MCs in innate and adaptive immunity. While physiologic functions of MCs have been increasingly recognized, they are notoriously known for their implication in allergies and other inflammatory diseases. Therefore,

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understanding the mechanisms of mediator secretion is of prime interest for therapeutic strategies. Significant advances have been made concerning the signaling steps initiated by the aggregation of the high affinity receptor for IgE (FcεRI).[1] This includes knowledge about the late events involved in the final membrane fusion steps during exocytosis. It has become clear that they rely on the evolutionary conserved machinery of SNARE fusion proteins as well as associated accessory proteins. This chapter describes and discusses the function of this molecular machinery.

REGULATED EXOCYTOSIS IN MAST CELLS

While all eukaryotic cells are capable of transporting and exocytosing proteins along the constitutive secretory pathway, some specialized secretory cells including MCs are capable of regulated exocytosis. They store a wide range of secretory products in cytoplasmic SGs, making them available for immediate release upon stimulation. Generally, exocytosis observed in neuronal, neuroendocrine or exocrine cells, involves the fusion of an individual SG with the plasma membrane (PM). For example, at the neuronal synapse this system is highly specialized and coupled to rapid endocytosis and regeneration of secretion-competent SGs within a second, which enables repetitive exocytosis.[2] By contrast, MCs are specialized to release virtually all granular content in a single stimulatory event, a process often referred to as degranulation.[1] Early ultrastructural and electrophysiological studies showed that degranulation involves compound exocytosis, which besides the fusion of granules at the PM, implicates fusion between granules, either in a multivesicular or sequential manner, thereby forming degranulation channels[3,4] (Fig. 1). At the PM fusion site, the proteoglycan matrix is expelled. Many, but not all,[5] of the mediators are tightly bound to this matrix, but are liberated by ion exchange due to the rise in pH and granule swelling.[6] The expelled proteoglycan core can sometimes be observed in the tissue surrounding MCs. MCs also exhibit piecemeal degranulation with progressive loss of granule particulate contents without any evidences for fusion.[7] Ultrastructural analysis has revealed the budding of small cytoplasmic granules that move towards the PM, leading to gradual emptying.[8] Progressive SG emptying could also result from transient fusion, often referred to as "kiss and run" fusion. Such transient fusion events have been shown to occur at a

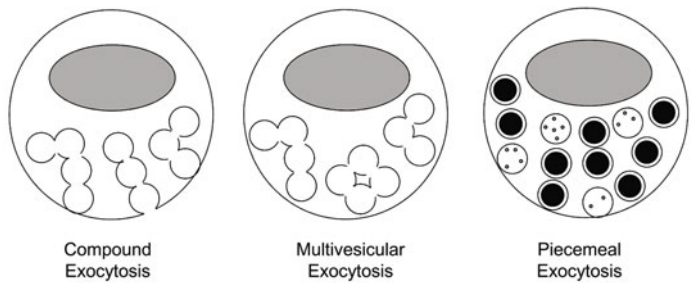

Figure 1. The different modes of exocytosis in mast cells are illustrated. Generally activation with a strong stimulus induces compound and/or multivesicular exocytosis. With some stimuli another mode of exocytosis called piecemeal degranulation can be observed, which involves gradual emptying of vesicular contents without any observable fusion event.

frequency of approximately 2:1 over full fusion in live imaged MCs.[9] MCs can participate in multiple cycles of activation for mediator release. However, in comparison to the very fast synaptic vesicle cycle, the regeneration of a fully reconstituted granular compartment can take up to 72 h.[10]

CHARACTERISTICS OF MAST CELL GRANULES

A peritoneal MC contains up to 1000 SGs occupying up to 40% of the cell volume.[11] While endo- or exocrine cells have specialized SGs, MCs or other cells of hematopoietic origin contain secretory lysosomes, which are organelles containing lysosomal hydrolases situated at the junction between the endo- and exocytic pathways.[12] Electron microscopic studies of MCs has revealed granular heterogeneity and allowed to the designation of Type I, II and III SGs differentiated on the basis of a specific marker and access to endocytotic tracers.[13] Type I SGs are multivesicular and could represent conventional lysosomes that release their content only under nonphysiological situations. Type II and III SGs could represent the compartment regulated by a physiological stimulus. Type II granules show an electron-dense core surrounded by membrane vesicles and are accessible to endocytotic tracers. Type III granules contain essentially electron-dense material and are not accessible by endocytotic tracers. The biogenetic relationship between these types of SGs remains to be elucidated. It has become clear that the granular compartment and proper loading with inflammatory mediators depends crucially on the expression of proteoglycans, as genetic targeting of the heparin biosynthesis pathway or the serglycin core in mice revealed severe defects in SG maturation.[5]

THE BASIC SECRETORY MACHINERY IN MAST CELLS

The SNARE Fusion Apparatus

MC degranulation is triggered by aggregating IgE bound to FcεRI. Recent studies have demonstrated that degranulation relies on the conserved mechanism of membrane fusion, implicating SNARE proteins initially discovered in neurons.[2] Each cell type expresses different combinations of SNARE members selectively distributed on intracellular membranes. Their selective pairing constrains trafficking and fusion between organelles or with the cell membrane. SNAREs contain an α-helical motif of about 60 aa, called a SNARE motif, four of which combine to form a stable tetrameric core trans-SNARE complex, the formation of which is energetically favored. This promotes vesicle docking with the target membrane and catalyzes fusion of the apposing membranes.[14] Functionally, SNAREs can be classified into v-SNAREs located on the vesicles and t-SNAREs located on the target compartment that can either be the PM or another vesicular compartment. Usually, a v-SNARE consists of a single central arginine-containing SNARE motif-containing vesicle associated membrane protein (VAMP) that pairs with central glutamine-containing t-SNARES, including a member of the synaptosome-associated protein-25 (SNAP-25, (neuronal) or SNAP-23, 29, 47 (ubiquitous) containing two SNARE motifs (light chains) and a Syntaxin subfamily member containing one SNARE motif (heavy chain).[15] SNAP-25 subfamily members can be replaced by SNAREs structurally related to the N-terminal half

of SNAP-25 (as one of the two light chains) and one member of the SNARE subfamily related to the C-terminal half of SNAP-25 (as the other light chain).[15]

MC exocytosis requires SNAREs, as blockade of their function with *N*-ethylmaleimide (NEM) strongly impairs degranulation.[16] NEM inhibits *N*-ethylmaleimide-Sensitive Factor (NSF), an ATPase that disassembles SNARE complexes. Transfection of a NSF-mutant unable to promote SNARE disassembly markedly inhibited exocytosis, indicating that SNARE disassembly is an integral part of the cycle that primes SNARE-mediated fusion.[17] MCs express a wide range of SNAREs. These include the t-SNAREs SNAP-23 and Syntaxins2, 3, 4 and 6.[18-20] Expressed v-SNAREs comprise the VAMP family members VAMP2, 3, 7 and 8.[18-20] The first protein characterized to function in degranulation was the PM-localized t-SNARE SNAP-23.[18] Introduction of blocking antibodies into permeabilized rat peritoneal MCs (RPMCs) potently inhibited stimulated exocytosis, a result confirmed in human primary MCs.[20] During exocytosis, SNAP-23 relocated from the PM along degranulation channels that formed in the interior of the cell, explaining a particular function in compound exocytosis.[18] Another study showed that overexpression of SNAP-23 in the RBL-2H3 mast cell line enhanced exocytosis.[21] Analysis of complex formation in mouse bone marrow derived MCs (BMMCs) and primary human MCs showed that complex formation between SNAP-23 and Syntaxin4 or VAMP8 increased during FcεRI-induced stimulation.[22] Concerning Syntaxin members, overexpression of the PM-localized Syntaxin4 in RBL-2H3 cells interfered with degranulation.[19] Furthermore, introduction of antibodies for Syntaxin4, but not of Syntaxin6, inhibited degranulation in permeabilized cells.[20] Like SNAP-23, Syntaxin4 formed enhanced complexes with SNARE partners VAMP8 and SNAP-23 after stimulation.[22] In addition, evidence for the implication of Syntaxin3 has been presented. This t-SNARE is primarily located on SGs and relocates to the periphery upon stimulation.[17] Furthermore, it is able to form complexes with other SNARE partners involved in exocytosis including SNAP-23 and VAMP7. Preliminary data in our laboratory show that siRNA-mediated knock-down of Syntaxin3 partly inhibits exocytosis. As Syntaxin3 is located on SGs, it might represent one of the t-SNAREs involved in granule-granule fusion.

Concerning VAMP proteins, four studies have implicated VAMP8, a v-SNARE initially shown to localize to early and late endosomes, confirming the close connection between the endocytic and the exocytic apparatus in MCs.[16,20,22,23] In several types of MCs, VAMP8 partially colocalizes with the SG markers, serotonin and mMCP-6 (tryptase). Tiwari et al[22] showed that BMMC from VAMP8-deficient mice inhibited degranulation as monitored by the release of histamine and β-hexosaminidase, but not chemokine/cytokine production. The role of VAMP8 was also confirmed in vivo, as absence of VAMP8 markedly inhibited histamine release in passive systemic anaphylaxis experiments. Similarly, introduction of a soluble recombinant VAMP8 protein into RBL-2H3 cells or of blocking antibodies into permeabilized human MCs markedly inhibited release of β-hexosaminidase or histamine.[20] Another study using VAMP8-deficient BMMC suggested that VAMP8 may further discriminate between granule subpopulations, as it may control the release of serotonin, cathepsin-D and β-hexosaminidase but not of histamine, which may localize to distinct SGs.[23] Distinct release of cathepsin D and histamine containing granules has also been reported when examining the role of syntaptotagminII.[24] However, these reports remain to be confirmed, as other studies have not seen independent secretion and localization of lysosomal and genuine MC granular markers.[19,20,22] Similarly, synaptotagminII-deficient BMMC did not show such a differential regulation.[25] MC activation resulted in the redistribution of VAMP8 from granular compartments to the

PM; and was associated with increased complex formation with PM-membrane localized SNAP-23 and Syntaxin4.[20,22] Besides VAMP8, other VAMPs have also been examined. Deficiency of VAMP2 and VAMP3, or introduction of blocking soluble recombinant proteins or antibodies, did not affect release, suggesting that they may not play a role.[20,22,23] This is consistent with the absence of colocalization with SG markers. However, it was found that ectopically expressed GFP-VAMP2 gradually fused with the PM following stimulation in RBL-2H3 cells.[26] Furthermore, a tendency to increased complex formation during stimulation can be observed both for VAMP2 and VAMP3 in the background of VAMP8-deficiency, suggesting that there may be compensatory effects. Differential results were obtained with VAMP7, a lysosomal marker. Initial experiments showed that VAMP7-CFP/YFP colocalizes with Syntaxin3 on SGs and relocates from granular structures to the PM after stimulation in RBL-2H3.[27] However, no effect on secretion was seen after introducing soluble VAMP7 into RBL-2H3 cells.[16] In human MCs, however, introduction of anti-VAMP7 markedly inhibited histamine release.[20] Taken together, these data define the role of SNARE fusion proteins in MC degranulation. The relocation of SNAP-23 maybe a particular feature of the compound exocytotic process by providing an efficient t-SNARE originally located at the PM.[18] The implication of a second t-SNARE, Syntaxin3, located on SGs may similarly increase the efficiency of compound exocytosis.

ACCESSORY PROTEINS IN MAST CELL MEMBRANE FUSION

In vitro reconstitution of the SNARE-mediated fusion process in liposomes exhibits a slow kinetics, suggesting that additional effectors are operative in vivo.[28] Besides increasing the efficiency of fusion, they may function in connecting the fusion machinery to cell signaling and second messengers. Several SNARE accessory proteins have been described in MCs.

NSF and α-SNAP

SNARE proteins were initially discovered by their ability to bind to soluble NSF attachment proteins (SNAPs) bridging the interaction to the ATPase NSF,[29] which mediates SNARE disassembly, the energy dependent step of fusion. Treatment of MCs with the NSF-inactivating compound NEM dramatically inhibits exocytosis, as does the expression of an ATPase-deficient NSF mutant, resulting at the same time in the accumulation of fusion-inefficient SNARE complexes.[17] This was confirmed by the inhibitory action of a dominant-negative α-SNAP unable to activate the ATPase.[16] These data point out the importance of SNARE disassembly or priming during degranulation.

SM Proteins

MCs also express SM (Sec1/Munc18) family members that bind Syntaxin proteins in an isoform-specific manner and which are known to play fundamental roles in various intracellular trafficking steps.[14] Munc18 (mammalian uncoordinated = unc-18) proteins, more specifically, are implicated in the regulation of exocytosis. Progress in understanding their function has come from knock-out studies of Munc18-1 and Munc18-3 isoforms. They demonstrated an essential role in fusion in various types of regulated-secretion competent cells.[14,30,31] The mechanisms could involve distinct

features that are in part coupled to their different modes of interaction with cognate SNARE proteins.[14,32] Binding to the closed "un-complexed" conformation of Syntaxin (containing like the SNARE complex a four-helix bundle composed of three N-terminal helices of the Habc domain and the SNARE motif) blocks SNARE assembly and could play a role in gating the initiation of the fusion reaction.[14] Such binding may also have a chaperone-like function, as expression of various Syntaxins was shown to decrease in the absence of Munc18 isoforms.[14] By contrast, binding to the trans-SNARE complex may more directly be involved in promoting fusion.[33] Evidence obtained from different systems suggest that Munc18 isoforms have additional functions in vesicle translocation, tethering and docking,[32] which may not necessarily relate to SNARE binding per se, but could involve interaction with other effectors such as Mints,[34] Doc2,[35] Granuphilin[36] and Cab45.[37]

MCs express the ubiquitous isoforms Munc18-2 and Munc18-3, while neuronal Munc18-1 has been detected so far only at the mRNA level.[38,39] Functional studies demonstrated a role for Munc18-2, which is localized on SGs and associates to Syntaxin3 and Syntaxin2. Ectopic expression of Munc18-2 and Munc18-2 peptides interfere with an effector loop inhibited MC exocytosis.[38] Similarly, siRNA-mediated knock-down of Munc 18-2 inhibited exocytosis.[40] This supports recent data obtained in patients carrying mutations in Munc18-2 that have revealed a deficiency in expression and stability of Munc18-2 and a consequent secondary down-regulation of Syntaxin11, causing familial hemophagocytic lymphohistiocytosis Type5 (FHL5) impairing lytic granule exocytosis.[41,42] In contrast to other immune cells,[43] so far no role has been demonstrated for the PM-localized and Syntaxin4-interacting isoform Munc18-3 in MCs.

Munc13-4

Recent studies also indicated a role of the Munc13 (mammalian unc-13) family members in mast cell degranulation.[29,44] The Munc13 family contains three brain-specific isoforms, Munc13-1, 13-2 and 13-3; and a nonneuronal isoform Munc13-4, which is expressed in the lung, spleen and hematopoietic cells.[44-46] Munc13 family members are active zone proteins at the synapse involved in vesicle priming. The finding that a mutated constitutively "open" Syntaxin1 can partially rescue the fusion block in Munc13-1/13-2 double KO mice suggests a role in the conformational transition of Syntaxin1.[44] Furthermore, the function of Munc13 in priming implies an interaction with the adaptor protein Rim which is able to bind Rab3 family members, both of which play a role in the regulation of fusion.[44] Mutations causing a loss of Munc13-4 function expressed in hematopoietic cells impair exocytosis of cytotoxic SGs responsible for familial FHL3.[47] In cytotoxic T cells, Munc13-4 has dual function.[48] In an early step, it promotes attachment of "exocytotic" vesicles generated from Rab11/Munc13-4-positive and Rab27A-positive endosomal vesicular pools; and then it primes fusion of genuine cytotoxic SGs with these "exocytotic" vesicles to secrete their content. Priming involves interaction of Munc13-4 with Rab27A (see also below). In MCs, both Munc13-4 and Rab27 isoforms colocalize on SGs, where Munc13-4 is anchored via its Munc-homology domain.[46] Over-expression of Munc13-4, but not mutated Munc13-4 enhances degranulation, suggesting a positive regulatory function.[45,46] The mechanism may involve the interaction with Doc2α, a calcium effector of exocytosis. Munc13-4 and Doc2α and Rab27A can form a tripartite complex. The importance of Doc2α is underlined by the marked inhibition of exocytosis in Doc2α-deficient BMMC.[35]

Rab GTPases

Rab proteins constitute a large family of small GTPases involved in intracellular trafficking, including control of membrane identity, vesicle budding, uncoating, motility and fusion.[49] In particular, Rab3 family members have been recognized as regulators of late steps in exocytosis. At the synapse, they are not essential per se, but function as gatekeepers of calcium-triggered exocytosis by a still poorly understood mechanism.[29] Some of their actions could relate to vesicle docking and priming via interaction with effectors such as Rim/Munc13-4 and rabphilin3A. In MCs, Rab3A, Rab3B and Rab3D are expressed when examined by PCR analysis.[50] Protein expression was confirmed for Rab3A and Rab3D and the latter was shown to translocate from SGs to the PM following stimulation.[50,51] Over-expression of Rab3D or a constitutively active GTP bound mutant, but not of Rab3A, revealed an inhibitory effect in antigen-stimulated RBL MCs.[50] Another study showed that over-expression of Rab3A also inhibited exocytosis.[52] The role of Rab3D has been challenged, because MCs from Rab3D-deficient mice have exhibited normal exocytosis and do not reveal any increase in granule size as shown for pancreatic zymogen granules.[53] However, the role of compensatory mechanisms by other Rabs has not been addressed.

Recently, the implication of another GTPase, Rab27, has been reported.[45,54] It exists in two isoforms (Rab27A/Rab27B) known to interact with a variety of Rab27-binding proteins such as Slac2 (synaptotagmin-like protein (Slp) family with tandem C2 Calcium-binding motifs) and Munc13-4, all functioning in membrane trafficking.[55] Interaction with Slac2 family members couples Rab27 function to the actin-based motor MyosinVa in melanocytes and neuroendocrine cells.[56] This mode of action does not apply to Rab27-deficient cytotoxic T cells (CTLs), which nevertheless are unable to release granular contents due to a docking defect.[47] Mutations in Rab27A are responsible for the FLH2 secretory phenotype in humans. This includes pigmentation defects as, unlike Munc13-4, Rab27A is also expressed in melanocytes. MCs express Rab27A and Rab27B isoforms, which localize to SGs.[45,54] However, degranulation defects are only observed in Rab27B- and more strongly, double Rab27-deficient cells. Similarly, defects in PCA were observed in Rab27B and double KO mice, while surprisingly, PCA was enhanced in Rab27A mutant mice.[45,54] Further functional analysis showed that Rab27B-deficient MCs exhibit mild SG clustering with a 10-fold increase of SGs in microtubule-dependent movement, suggesting that Rab27B action could regulate the transition from microtubule to actin-based motility.

Complexins

Recently described SNARE-interacting effectors are the cytosolic proteins, complexins I and II.[14] Complexins preferentially bind to assembled SNARE complexes, which then blocks completion of assembly and hence transfer of force required for fusion pore expansion, leading to a "superprimed" state ready for immediate fusion.[57] It is thought that the calcium sensor synaptotagmin finally triggers fusion.[14,44] Calcium binding to the tandem C2A and C2B domain induces simultaneous SNARE and membrane phospholipids binding thereby reversing the complexin block, in addition to the induction of fusion-promoting membrane phospholipids. Complexin may additionally participate in fusion activation through its N-terminal domain binding to a membrane-proximal v-SNARE sequence, thereby pulling the complex closer to the membrane.[57]

MCs express Complexin II but not Complexin I.[58] Complexin II functions as a positive regulator, as siRNA-mediated knock-down markedly suppressed IgE-induced degranulation.[58] The sensitivity of the degranulation process for calcium was decreased, as knock-down cells required higher extracellular calcium concentrations for exocytosis. Immunofluoresence studies showed that during stimulation, Complexin relocated from the cytoplasm to the PM, where it might interact with trans-SNARE complexes. Indeed, it binds tightly to a complex composed of Syntaxin3, SNAP-23 and VAMP8 or 2.[40] Taken together these studies indicate that Complexin II in MCs, rather than acting as a fusion clamp, may promote fusion by enhancing its calcium sensitivity. Whether this implies concerted action with a synaptotagmin family member remains an open question.

Synaptotagmins

Synaptotagmins (Syts) comprise a small family of single TM proteins involved in calcium-dependent regulation of exocytosis.[29] They contain tandem calcium binding C2 domains (termed C2A and C2B) binding three and two calcium ions, respectively. The affinity of binding may vary considerably between isoforms and can be affected by the presence of Syt binding partners. Calcium binding promotes oligomerization and membrane phospholipid binding,[44] thereby allowing simultaneous binding to two membranes. It also promotes binding to the SNARE complex, allowing the formation of a quaternary SNARE-synaptotagmin-calcium-phospholipid complex, thereby connecting the basic fusion machinery to calcium-triggered exocytosis.[44]

MCs express several Syt family members (SytI, II, III and IX).[24,59-62] Initial siRNA knockdown studies showed that deficiency of SytII did not ostensibly affect histamine release, but rather blocked release of cathepsin D suggesting that it acts to prevent fusion of genuine lysosomes, but not SGs.[24] However this finding has been challenged, as BMMC grown from SytII-deficient mice showed a severe defect in both lysosomal β-hexosaminidase and histamine release.[25] Unlike in neurons, SytII, which has a perigranular location, does not affect spontaneous granular release. SytIII was found to function as a critical regulator of the perinuclear endocytic recycling compartment (ERC) and could possibly regulate SG size,[60] while SytIX functions in protein export from the ERC to the cell surface.[61] These data suggest that nonneuronal Syts interfere with distinct steps of membrane trafficking in MCs, along the endo- and exocytic pathway.

SCAMPs

A further accessory protein that regulates the fusion competent state are secretory carrier membrane proteins (SCAMPs), a family of ubiquitous membrane proteins of transport vesicles. They are highly expressed in organelles undergoing regulated exocytosis such as synaptic vesicles, but also mast cell SGs.[63] They are tetraspanins with the short segment (E peptide) linking the second and third TM domain being the most highly conserved element and unique to SCAMPs.[64] MCs express SCAMP1 to SCAMP3 isoforms, with SCAMP1 and SCAMP2 being most prevalent.[65] They localize to SGs and vesicles, but a small fraction is also found at the PM colocalizing with Syntaxin4 and SNAP-23. Introduction of the E-peptide potently inhibits exocytosis in permeabilized MCs.[65] Further studies have indicated that it acts likely at the final fusion step subsequent to SNAP-23 relocation and ATP-dependent priming.[64,66] In agreement, genetic deficiency of SCAMP2 causes an apparent defect in forming stable fusion pores.[63] SCAMPs may act at a step that

couples Arf6-stimulated phospholipase D (PLD) activity to formation of fusion pores, as the peptide also inhibits PLD activity.[64] Furthermore, in PC12 cells it has been shown that a SCAMP2 mutant with decreased Arf6-binding recapitulated the inhibitory effect. This could be in agreement with the proposed implication of PLD in MC exocytosis.[67,68]

Role of the Cytoskeleton in Secretion

MC degranulation involves extensive cytoskeletal reorganization with dissolution of the subcortical actino-myosin complex and formation of F-actin ruffles.[69] In parallel, microtubules stretch out into the lamellipodia formed during the stimulation process.[70] Furthermore, SGs are embedded in a filamentous network of cytoskeletal structures with hook-like structures.[71] The cytoskeleton could provide an appropriate scaffold for signaling proteins and the attachment of cellular compartments and proteins of the fusion machinery. The actino-myosin contractile system or tubulin-based motors could be important for the mobilization and extrusion of SG content.[72] On the other hand, cortical actin could represent a substantial barrier. Convincing evidence for the requirement of cortical actin depolymerization has come from studies combining atomic force and confocal microscopy, which showed that granule fusion occurs only at PM sites devoid of cortical actin.[73] Similarly, treatment with actin-depolymerizing drugs or ectopic expression of a constitutively active mutant of RhoA, Rac1 or CDC42 promoting actin remodeling enhances secretion, while dominant-negative forms inhibited secretion.[69,74] However, other functional consequences of these treatments, notably interference with early cellular signaling pathways, have been described, suggesting a complex relationship.[74,75]

Concerning the tubulin network, treatment with microtubule destabilizing or stabilizing drugs affected secretion in MCs.[38,76] In stimulated MCs, SGs identified by Munc18-2-staining were excluded from F-actin-containing ruffles but appeared to be aligned along newly formed microtubular tracks, suggesting microtubular transport mechanisms.[38] In agreement, live imaging of SGs revealed bidirectional movement in both resting and activated cells adjacent to microtubules; and the rate and extent of exocytosis was inhibited by the microtubular depolymerizing drug Colchicine.[77] A recent study showed that degranulation can be separated into two independent pathways based on the following: i) the microtubule-dependent and Fyn/Gab2/RhoA-regulated translocation of CD63-GFP-positive SGs to the PM occurred in a calcium-independent manner; and ii) the actual fusion required increases in intracellular calcium concentrations.[76] Thus, it is possible that, similar to other hematopoietic cells with a more locally restricted release,[78] the microtubular system also plays an important role in the secretory process of MC. These data, however, contrast with studies which do not reveal significant SG movement during exocytosis in RBL cells.[9] Similarly, RPMCs with a highly differentiated SG compartment rather show an inward movement of PM components such as SNAP-23.[18] Still, the absence of movement does not exclude a coordinated action between the cytoskeleton and the fusion apparatus, for example, to guide the extensive membrane rearrangements and to provide pulling force.

Taken together, at present it is still difficult to propose a cohesive model on the implication of cytoskeletal elements in the late steps of secretion. This is largely due to the complexity of the molecular pathways involved, which not only impinge on the late steps of exocytosis, but on a whole variety of cellular processes. Nevertheless, accumulated data suggests that cytoskeletal functions are an integral part of the fusion process in MCs.

COUPLING OF THE FUSION MACHINERY TO SIGNAL TRANSDUCTION FOR EXOCYTOSIS IN MAST CELLS

The Role of Kinases and Phosphatases

Aggregation of FcεRI is a potent stimulus for degranulation. This allows docking of nonreceptor tyrosine kinases, thus launching an activation cascade.[1] Protein phosphorylation-dephosphorylation represents an important regulatory mechanism in the late steps of MC exocytosis. Several fusion proteins have been shown to become phosphorylated by Ser-Thr kinases, including PKC, CaM kinaseII, PKA, Casein kinaseII. Phosphorylation could play a variety of functions in the fusion process including regulation of the activity state of SNARE proteins[79] or a direct interference in the fusion process.[80]

In MCs, activation of protein kinaseC (PKC) represents an essential signal for secretion. A calcium-dependent isoform, PKCβ and a calcium-independent isoform, PKCδ, have been implicated using depletion-reconstitution studies.[81] The role of PKCβ was confirmed in PKCβ-deficient MCs,[82] while PKCδ-deficient MCs actually showed enhanced exocytosis.[83] These apparent differences may be explained by indirect effects of PKCδ on negative regulatory mechanisms. Regardless, PKC clearly regulates late steps in exocytosis. Molecular targets include myosin light and heavy chains necessary for the reorganization of the actino-myosin cortex during secretion.[84] PKC also phosphorylates Munc18-1 within its second domain, which prevents Syntaxin binding.[85] Similarly, phosphorylation of Munc18-3 by PKC reduces its affinity for Syntaxin2 and 4 in platelets[86] and thus could regulate the amount of available fusion-competent Syntaxin. PKC also phosphorylates SNAP-25 and Syntaxin4 in vitro.[87] In MCs, about 10% of SNAP-23 becomes transiently phosphorylated during degranulation on Ser^{95}/Ser^{120} within its cysteine-rich linker region.[88] While PKC was a possible effector, recent data have implicated IκB kinase (IKK), generally known to phosphorylate IκB, which induces nuclear translocation of the NF-κB transcription factor.[89] IKK-deficient MCs have markedly impaired degranulation and anaphylactic responses when reconstituted into MC-deficient mice. This was independent of NF-κB translocation. In vivo, IKK phosphorylated residues Ser^{95}/Ser^{120} and reconstitution with phosphorylation-mimetic mutant into IKK-deficient MCs partially restored, while over-expression of phosphorylation mutants inhibited IgE-stimulated exocytosis.[88,89] Mechanistic analysis showed that, in IKK-deficient cells, SNAP-23 formed fewer complexes with Syntaxin4, VAMP2 and VAMP8, suggesting that phosphorylation on Ser^{95}/Ser^{120} is required for complex formation.

A different calcium-regulated kinase activity present in Rab3D-containing immunoprecipitates was responsible for specific phosphorylation of Syntaxin4. In contrast to SNAP-23, the phosphorylation of Syntaxin4 decreased its SNAP-23 binding capacity, although so far this effect has only been studied in vitro.[79]

Besides PKC, SNARE Kinase (SNAK) could also be a regulator of exocytosis in MCs.[90] SNAK phosphorylates SNAP-23 at Ser-Thr residues thereby increasing the stability of newly synthesized protein. SNAK-dependent phosphorylation could thus indirectly promote SNARE complex formation by increasing the pool of available SNAP-23.

Protein phosphorylation is regulated by a dynamical balance between the action of kinases and phosphatases. Phosphatases likely also regulate fusion in MCs.[91] Inhibitors of phosphatase PP1 and PP2a reduced secretion even when bypassing early-receptor-mediated signals.[92] PP2A is recruited to the PM in a manner that correlates with the kinetics of secretion. Subsequent studies in RBL-2H3 cells have demonstrated that both PP1 and

PP2A transiently associate with cortical myosinII, suggesting a role in the cytoskeletal rearrangements of the actino-myosin cortex.[93] A crucial target for phosphatase activity could be the PKC-dependent phosphorylation sites in myosin light and heavy chains. Another phosphatase implicated in membrane fusion includes megakaryocyte cytosolic protein tyrosine phosphatase 2 (MEG2) localized to SG, whose over-expression results in the formation of large granules.[94] MEG2 is known to dephosphorylate NSF on a key tyrosine residue important for its activity.[95]

TARGETS OF CALCIUM

Although it was realized more than 50 years ago that calcium is an essential intracellular messenger for secretion in neurons and somewhat later in MC,[96] the molecular targets remained obscure for many years. The synaptotagmin calcium sensors have now emerged as prime candidates. They contain the conserved tandem calcium and phospholipid-binding C2 domains (C2A and C2B).[44] Calcium binding promotes the formation of a multi-molecular complex thought to finally trigger together with SNARE assembly and complexin the final fusion.

Another calcium sensor is calmodulin (CaM) interacting with numerous proteins that have a role in secretion.[97] Results of inhibitor studies in MCs range from no effect to an inhibitory effect on secretion; and are difficult to interpret with respect to the function of this sensor.[98,99] In MCs, potential targets include myosin light chain kinase (MLCK),[100] CaM kinaseII which phosphorylates nonmuscle myosin,[101] Syntaxin3[102] and PLD.[67] CaM binds also VAMP2.[103] CaM is thought to activate VAMP2 by liberating it from its lipid-bound state upon binding.[103] In agreement, microinjection of a VAMP2-derived peptide that blocks CaM-binding into chromaffin cells resulted in inhibition of exocytosis.[103] However, calmodulin-binding has not been observed for VAMP8, which has been described as a major v-SNARE in MC exocytosis.

In addition, a variety of other molecules that coordinate the secretory fusion process are also calcium sensors. These include Munc13-4 and Doc2α, which are also known to express calcium-binding C2 domains.[44]

Evidence for Lipid Raft Domains in Mast Cell Exocytosis

Lipid rafts are dynamic assemblies of cholesterol and sphingolipids, which form a separate liquid-ordered phase in membranes that are enriched in discrete subsets of proteins. At the PM, they regulate signal transduction by providing concentrated platforms for signaling proteins.[104] Lipid rafts are also found in biosynthetic and endocytic pathways.[105] Raft-dependent interactions may also play a role in exocytosis, as treatment with cholesterol-depleting agents considerably affected regulated secretion in several cellular systems.[106] Moreover, both vesicular and PM fusion proteins become enriched in lipid rafts, although the nature of this compartment is still somewhat under dispute.[107,108] SGs preferentially dock and fuse at these identified cholesterol-dependent clusters. Thus, similar to the platforms for transmembrane signaling, such domains may also form functional entities for fusion. In MCs and when examined by sucrose gradient fractionation, SNAREs were either excluded (Syntaxin2), equally distributed between raft and nonraft fractions (Syntaxin4, VAMP8, VAMP2), or selectively enriched in rafts (Syntaxin3, SNAP-23).[109] The accessory protein Munc18-2 and 18-3 were found in nonraft fractions.

However, small amounts of Munc18-2 also consistently distributed into rafts. Cognate SNARE complexes of Syntaxin3 with SNAP-23 or VAMP8 were also enriched in rafts, while those of Syntaxin3 with Munc18-2 were excluded. A separate study[110] also found a selective enrichment in raft fractions of SNAP-23/Syntaxin4 and Syntaxin4/VAMP2 complexes. In addition, quantitative analysis showed that raft-associated Syntaxin4 and VAMP2 increased about twofold in FcεRI-stimulated cells, forming complexes containing Syntaxin4, VAMP2 and phospho-SNAP-23. Taken together, these data point out an important role of the membrane lipid environment in MC exocytosis.

CONCLUSION

MC-triggered compound exocytosis is fundamental in immunity, but also plays a detrimental role in inflammatory diseases. Like other secretory cells, MCs use specific and highly regulated, secretory machinery that includes cognate SNARE membrane fusion proteins as well as various accessory regulatory proteins. These proteins and the evidence for implication are summarized in Figure 2. Together, this machinery

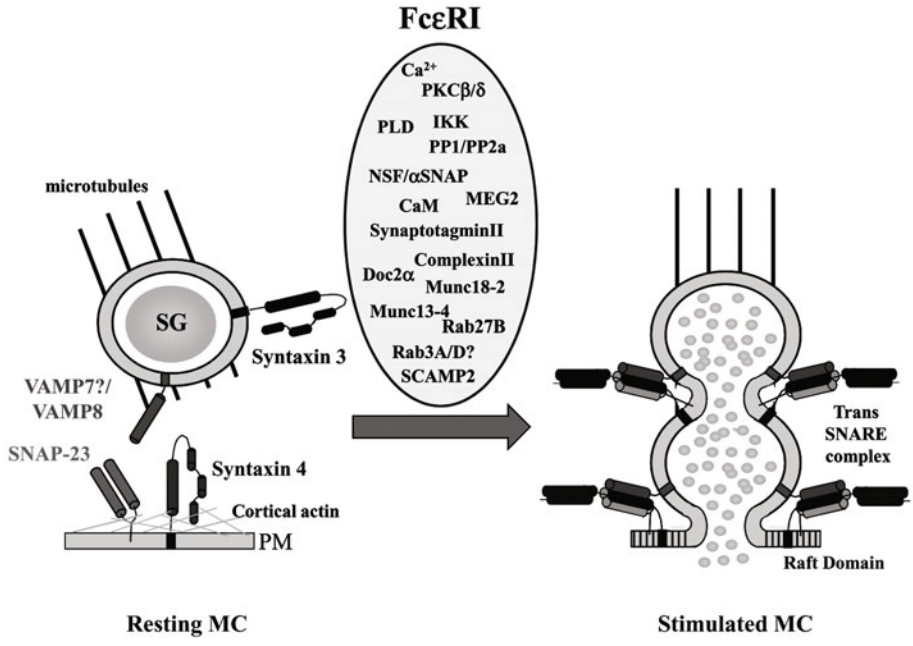

Figure 2. Molecular mechanisms of exocytosis in mast cells. Mast cells use a specific and highly regulated secretory machinery that includes cognate SNARE membrane fusion proteins such as the vesicle-associated membrane proteins (VAMP7/8), the plasma membrane-associated syntaxin4 (eventually Syntaxin3 in case of granule-granule fusion) and SNAP-23. These enable formation of a stable multi-molecular complex that energetically favors the merger of lipid membranes. SNARE complex formation is regulated by multiple accessory proteins that may either directly assist the fusion to increase its efficiency, or serve as signaling effectors that connect to early stimulatory events and cytoskeletal rearrangement. For detailed explanation of their mechanism refer to the text. Note that fusion at the PM occurs preferentially in lipid rafts.

coordinately functions to protect the organism from unwanted release of potentially dangerous substances; but also promotes release upon arrival of a physiologic signal at the surface. In disease, besides inappropriate triggering, MCs often show a hyper-activated phenotype, translating into inappropriate regulation. Further research efforts will continue to focus on the molecular understanding of this machinery.

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CHAPTER 8

AN EMERGING ROLE FOR THE LIPID MEDIATOR SPHINGOSINE-1-PHOSPHATE IN MAST CELL EFFECTOR FUNCTION AND ALLERGIC DISEASE

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Abstract: Sphingosine-1-phosphate (S1P) plays important roles regulating functions of diverse biological systems, including the immune system. S1P affects immune cell function mostly by acting through its receptors at the cell membrane but it can also induce S1P receptor-independent responses in the cells where it is generated. S1P produced in allergically-stimulated mast cells mediates degranulation, cytokine and lipid mediator production and migration of mast cells towards antigen by mechanisms that are both S1P receptor-dependent and independent. Even in the absence of an antigen challenge, the differentiation and responsiveness of mast cells can be affected by chronic exposure to elevated S1P from a nonmast cell source, which may occur under pathophysiological conditions, potentially leading to the hyper-responsiveness of mast cells. The role of S1P extends beyond the regulation of the function of mast cells to the regulation of the surrounding or distal environment. S1P is exported out of antigen-stimulated mast cells and into the extracellular space and the resulting S1P gradient within the tissue may influence diverse surrounding tissue cells and several aspects of the allergic disease, such as inflammation or tissue remodeling. Furthermore, recent findings indicate that vasoactive mediators released systemically by mast cells induce the production of S1P in nonhematopoietic compartments, where it plays a role in regulating the vascular tone and reducing the hypotension characteristic of the anaphylactic shock and thus helping the recovery. The dual actions of S1P, promoting the immediate response of mast cells, while controlling the systemic consequences of mast cell activity will be discussed in detail.

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INTRODUCTION

Sphingosine-1-phosphate (S1P) is a lipid mediator, formed by phosphorylation of sphingosine (SPH), that is involved in multiple physiological and pathological processes. Of the vast numbers of processes known to be regulated by S1P, vascular development, vascular permeability, angiogenesis, tumorigenesis and lymphocyte trafficking have been extensively explored in the last decade.[1-4] The involvement of S1P in these physiological and pathophysiological processes results from its ability to modulate important cellular events such as chemotaxis, cytoskeletal changes, survival and proliferation.[5,6] In the last few years, it has become clear that the involvement of S1P in immunological responses is not restricted to its regulation of lymphocyte trafficking but extends to the regulation of immune cell function.[7] In mast cells, S1P regulates chemotaxis and mast cell effector responses such as degranulation, cytokine and lipid mediator production.[8] A novel picture of S1P as functioning beyond its role as a regulator of mast cell function in allergic disease is emerging, with the new discovery of its role in termination of some allergic reactions.[9]

S1P AND ITS TARGETS

The generation of S1P is mediated by the enzymatic activity of two cytosolic sphingosine kinase isoforms (SphK1 and SphK2) and occurs mostly at the membrane where the substrate resides and to where these enzymes are translocated after activation.[6,10,11] S1P has enough aqueous solubility to partition in the soluble fraction and to diffuse between intracellular membranes.[5] However, the site of action is not merely intracellular.[6] S1P can be exported out of cells via specific transporters and specifically bind to any of five G-protein coupled receptors (S1PR1-5) present in the cells of origin or in neighboring cells, engaging distinct signaling pathways as mediated by their coupling to specific isoforms of heterotrimeric GTP-binding proteins.[5,6,12,13] Thus, engagement of any receptor that promotes activation of SphKs and generation of S1P can potentially cause trans-activation of S1P receptors, enhancing the signaling repertoire and creating another wave of signals. Beyond its effect on S1PRs, the acute generation of S1P can also affect certain cell functions independently of its receptors[6,10] by either binding and modifying putative intracellular targets or by affecting the relative levels of other lipid products, particularly SPH and ceramide whose effects generally oppose those of S1P[1,5,6] (Fig. 1). Recently, histone deacytylase was described as the first known intracellular target of S1P. S1P generated in the nucleus, via the translocation and activation of SphK2, was found to bind histone deacetylase, inhibiting its activity and thus enhancing gene transcription.[14]

REGULATION AND FUNCTION OF S1P IN TISSUES AND CIRCULATORY FLUIDS

The levels of S1P inside cells and the interstitium of tissues are usually low in homeostatic conditions, due to irreversible degradation of S1P by a S1P lyase[15,16] and to its dephosphorylation to SPH by two specific S1P phosphatases (SPP1 and SPP2)[17] (Fig. 1) present in intracellular membranes and/or at the plasma membrane facing the extracellular environment. However, local increases in S1P levels may occur under certain conditions,

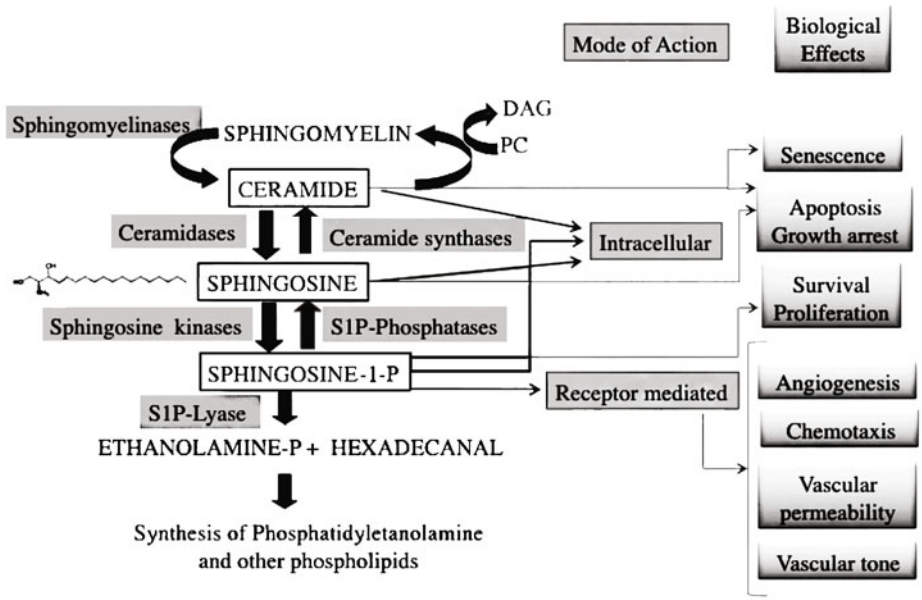

Figure 1. Sphingolipid metabolism and biological functions of sphingolipid derived molecules. Ceramide, sphingosine and sphingosine-1-phosphate are lipid mediators derived from complex sphingolipids by the action of several enzymes acting on cellular membranes. Sphingomyelinases cleave the phosphocholine group of sphingomyelin to yield ceramide; ceramidases then cleave ceramide to form sphingosine and sphingosine kinases phosphorylate sphingosine to form sphingosine-1-phosphate (S1P). These enzymes can be selectively or sequentially activated by various stimuli. The sphingolipid metabolites are interconvertible: S1P can be dephosphorylated to form sphingosine by S1P phosphatases and sphingosine acetylated by ceramide synthase. S1P can also be irreversibly degraded by a S1P lyase and the products of its activity used for the resynthesis of lipids. While ceramide and sphingosine were shown to induce apoptosis and cell cycle arrest, S1P prevents apoptosis and promotes cell growth. S1P mediates numerous cellular responses, not only by acting inside cells, but by binding a family of five plasma-membrane G-protein-coupled receptors (known as S1PR1–S1PR5) that are differentially expressed by most cells and mediate multiple functions. S1P produced intracellularly can be exported out of cells by specific transporters, or to a lesser extent, it may be generated in the extracellular space.

inducing responses in tissue resident or circulating immune cells (which express various combinations of S1PRs) affecting physiological processes.[7] For example, during the early stages of inflammation, increases in S1P at the site of inflammation can activate the S1PR1 in T cells[18] and hematopoietic stem and progenitor cells,[19] preventing their exit into the afferent lymphatics and causing T-cell retention in secondary lymphoid tissues and proliferation of tissue-resident myeloid cells, respectively. Furthermore, elevation of local S1P can be involved in modulating the type of immune response by skewing the phenotype of lymphocytes, dendritic cells and macrophages.[7] How S1P is elevated in tissues is still unclear; nonetheless, it is known that blood cells,[20] and as we will discuss later, mast cells, are likely candidates for increasing S1P in the tissue environment since they can produce and release large quantities of S1P when stimulated.

In contrast to the low levels of S1P in tissues, S1P concentrations in plasma and lymph are well above the binding affinity of S1P for its receptors (although it is unclear what is the "effective" concentration of S1P in circulation). Free S1P may be at least 40

times lower than the total S1P concentration because it is mostly bound to albumin or lipoproteins, but the bound form can be also physiologically active.[21,22] The S1P-carrier proteins may provide a sink for excess S1P and/or a regulatory mechanism for protecting S1P from degradation and for delivering S1P to specific cells.[21,22] Recent data has uncovered that the high levels of S1P in circulatory fluids play a role in two important physiological processes: the maintenance of endothelial barrier integrity and lymphocyte recirculation between lymph nodes and periphery. Deletion of both SphK1 and SphK2 in an inducible mouse model renders these "S1P-less mice" with enhanced vascular permeability and induces lethality in response to mediators that induce vessel permeability.[23] Resistance to lethality can be restored by transfusion of wild type erythrocytes,[23] the cells responsible for maintaining high levels of S1P in blood.[24,25] The proposed mechanism for the protective effect by S1P in the vascular barrier is its regulation of S1PR1 activity in endothelial cells.[23] S1PR1 is normally abundant in these cells and S1P engagement of this receptor tightens endothelial adherens junctions[26] protecting endothelium integrity. On the other hand, targeted deletion of SphK1 and 2 in the lymphatic endothelium, which results in ablation of the high concentrations of S1P in lymph but not in blood, causes impaired egress of lymphocytes from the lymph nodes.[27] Previous findings also showed that ablation of the tissue to blood/lymph S1P gradient by inhibition or genetic deletion of S1P lyase prevents the exit of lymphocytes from the lymph nodes,[15,28] and that the presence of S1PR1 in lymphocytes is required for their egress from lymph nodes.[29,30] Collectively, the findings argue that a major determinant driving the movement of lymphocytes out of the lymph nodes is the S1PR1-dependent sensing by lymphocytes of an S1P gradient that exists between the lymph node (low levels of S1P) and the lymph (high levels of S1P).

THE SPHK/S1P/S1PR AXIS AND MAST CELLS

From the brief discussion above, it is apparent that production and degradation of S1P is tightly and dynamically regulated. This suggests that its export to the extracellular space, its levels in the extracellular space and circulatory fluids and the regulation of cell surface expression of the multiple S1PRs must be critical in the shaping of a given physiological response. In this chapter we will summarize the recent knowledge on the regulation of S1P production and the function of S1PRs in mast cell biology and in the allergic responses. We now know that engagement of the high affinity receptor for IgE (FcεRI) induces the activation of SphK1 and SphK2 and the generation of S1P.[8] We also know that the activities of SphKs are key to the activation and expression of S1PRs on mast cells, however, it should be recognized that SphKs are also essential to FcεRI-mediated mast cell activation independently of S1PR.[8,31] When activated, mast cells secrete S1P into the extracellular space and produce gradients of S1P within resident tissues that may contribute to the inflammatory response and tissue remodeling.[32-34] Moreover, dysregulation of S1P can affect the differentiation and responsiveness of mast cells and thus it may increase mast cell responses in health and (allergic) disease.[31,35] S1P also seems to play a vital role in overcoming the severe (patho)physiological responses seen during anaphylaxis.[9] Interestingly, its production in a nonhematopoietic compartment was found to be essential for the recovery from anaphylaxis. This indicates that the site of action for S1P may determine its role as a pro- or anti-allergic molecule.

S1P IS GENERATED BY ACTIVATED MAST CELLS

FcεRI Activates SphKs

It is well recognized that antigen-induced aggregation of IgE bound to FcεRI on mast cells elicits multiple biochemical events culminating in mast cell degranulation and the de novo synthesis and secretion of cytokines and lipid mediators.[36] One of the biochemical events that occurs downstream of FcεRI engagement is the activation of SphKs with a consequent increase in intracellular S1P levels, as first shown in RBL-2H3 cells, [37] This finding was later confirmed in nontransformed cultured mast cells, such as bone-marrow derived mast cells (BMMCs), liver-derived mast cells and human mast cells.[31,33,38,39] Engagement of FcεRI elicits the partitioning of this receptor to lipid rafts where sphingolipids, such as SPH (the substrate for SphK), are enriched. The Src kinases Lyn and Fyn, which initiate early FcεRI signaling, are needed for the translocation of SphKs to lipid rafts and for their activation.[33,40] SphKs interact with Lyn and Fyn, but not with Src or other tyrosine kinases such as Syk and the activation of SphK1 and 2 depends on the presence of these tyrosine kinases and their downstream signals. Fyn-deficiency caused a complete ablation of SphK activation. [33,40] Further support for the importance of Fyn in SphK activation was provided when Fyn expression was restored, which rescued the activation of SphK1 and SphK2.[33] The adaptor protein Gab2 and the lipid kinase PI3K, as well as other undefined signals, were also found to be important for the activation of SphKs downstream of Fyn kinase.[33] In comparison, Lyn-deficient mast cells showed a delay in the activation of SphK by IgE/Ag,[40] yet these cells recovered to achieve an almost normal SphK activation with time suggesting that Lyn may not be essential for the activity of SphKs but may play a role in facilitating their activation. It has been suggested that PLD 1 activity is also involved in both FcεRI-[39] and FcγRI-mediated SphK1 activation[41] in mast cells and human myeloid cells, respectively. This view is consistent with previous findings[42] as well as our own (AO and JR unpublished observation), that PLD activation is Fyn-dependent in mast cells. This suggests that PLD is also likely to contribute to the activation of SphKs. However, the specific mechanistic detail of how PLD may contribute to SphK activation and the relationship to the translocation/activation of SphK1 and 2 in mast cells is not entirely clear.

Role of SphKs in Mast Cell Biology and Function

To directly address the importance of each of these isoforms in mast cell function, we derived mast cells from embryonic liver progenitors of mice deficient in SphK1, SphK2 or both,[31] since the double deficiency in SphK1 and 2 is embryonic lethal.[43] Although our experiments with BMMC from SphK1 and SphK2 -deficient adult mice showed clearly that SphK2 was the major producer of S1P in these cells,[31] mast cell degranulation was not consistently diminished in all tested cultures. This led us to believe that SphK1 and 2 might have some redundant functions. Unlike BMMCs derived from adult mice, mast cells derived from liver progenitors of SphK2-deficient embryos consistently showed a reduction in the extent of the degranulation response and in the production of various cytokines and eicosanoids. In contrast, mast cells derived from liver progenitors of SphK1-deficient embryos showed no impairment in degranulation, whereas cells deficient in both SphK1 and SphK2 had the same impairment as SphK2-deficient mast cells.[31] The robust and consistent defect seen in the degranulation response of mast cells derived from

the SphK2-null embryos clearly differed from the more mild and variable result seen in SphK2-null BMMCs derived from adult mice. We interpreted these results to reflect a possible compensatory or epigenetic change present in the mast cells of SphK-deficient adult mice (as discussed in the next paragraph), although another possibility is that the two mast cells populations are not phenotypically comparable. This latter possibility is not unexpected as the mast cell phenotype is known to vary depending on the environment they populate in vivo, or the growth factors used in vitro.[44] Thus, certain populations of mast cells might differ in their use of SphK1 or SphK2 for compartmentalized or general S1P production. In agreement with the view that the microenvironment may be a determinant in how SphKs are used, we now have evidence (unpublished observation) that peritoneal mast cells from either SphK1 or SphK2-deficient adult mice have impaired degranulation responses (although again, this result was more variable in the SphK2-deficient peritoneal mast cells). Thus, it is plausible that the relative importance of SphK1 and SphK2 depends on the type of mast cell (Table 1). This is also consistent with the findings on the role of SphK1 and SphK2 in human mast cells. In human bone marrow-derived mast cells only SphK1 mRNA was found and silencing of this isoform by antisense oligonucleotide caused a reduction in degranulation.[39] In $CD34^{+}$-derived human mast cells [33] and in cord blood derived human mast cells (CBMC)[38] both SphK1 and 2 were present and could be activated by FcεRI engagement. Silencing of SphK1 and SphK2 by RNAi in the latter cells and in the human mast cell line LAD2[38] showed that SphK1 was involved in degranulation, migration toward antigen and secretion of the chemokine CCL2/MCP1, whereas silencing of SphK2 reduced cytokine production (Table 1).

Inconsistencies in Uncovering the Role of Sphks in Mast Cells Using Silencing RNA and Genetic Deletion Approaches

Apart from the possible heterogeneity of the mast cells in those studies, there are other possible technical considerations when studying the relative involvement of the SphK isoforms. Study of BMMCs derived from SphK1 and SphK2-deficient mouse models, which were generated independently of those we study, showed normal degranulation responses to FcεRI stimulation.[45] Yet, the same report shows a reduction in the degranulation of SphK1-deficient BMMCs where SphK1 expression was silenced in vitro or in vivo by siRNA (Table 1). Thus, it is important to consider the possible limitations of employing RNAi silencing or genetic deletion strategies in interpreting the results. The difference observed between the SphK-deficient BMMCs generated by genetic deletion versus RNAi silencing might be most easily ascribed to off-target effects of siRNA or compensatory mechanisms from genetic deletion. The in vivo use of siRNA is also inherently nonspecific and would be expected to affect multiple populations of cells, not just mast cells. Alternatively, the genetic deletion could cause changes in the environment that may impose epigenetic changes or compensatory mechanisms in bone marrow progenitors. On this latter point, the SphK2-deficient mice used in our studies had elevated levels of S1P in plasma whereas the SphK1-deficient mice had low levels of circulating S1P.[43,46,47] As we demonstrated[31] this had a marked effect on mast cell responsiveness when mice were systemically challenged, since SphK2-deficient mice showed normal histamine release. This was unexpected given that fetal-liver derived SphK2-deficient mast cells showed defective degranulation. However, this is consistent with the recent report that S1P can regulate the development of human mast cells derived from cord-blood progenitors into a mast cell type more akin with a skin-type mast cell.[35]

Table 1. Reported role of SphK1 and SphK2 isoforms in mast cell responses in vitro and in vivo, using different experimental approaches

SphK Isoform	Type of SphK Alteration	Type of MC	Effect in Vitro	Effect on PCA (in vivo)	Effect on PSA (in vivo)
SphK1	siRNA, Antisense oligo	BMMC*	↓Degranulation[45] ↓ Ca response[45] ↓ Cytokine[45]	↓[45]	↓[45]
		Hu MC*	↓Degranulation[38,41] ↓ Ca response[41] ↓ Cytokine[38]		
	Knockout	BMMC	Normal[31]	Normal (unpublished)	↓Histamine[31] ↓ Recovery[9] (Strong response)
		LDMC*	Normal[31]		
		PMC*	↓Degranulation (unpublished)		
SphK2	siRNA	BMMC	Normal[45]	Normal[45]	
		Hu MC	↓ Cytokine[38]		
	Knockout	BMMC	↓Degranulation[31] Normal[45]	↓ (unpublished)	Normal histamine[31] ↑Recovery[9] (Poor response)
		LDMC	↓Degranulation[31] ↓ Ca response[31] ↓ Cytokine[31]		
		PMC	↓Degranulation (unpublished)		

*BMMC: murine bone marrow-derived cultured mast cells; Hu MC: human cultured mast cells (cord blood- or CD4+-derived); LDMC: mouse fetal liver derived mast cells; PMC: mouse peritoneal mast cells.

Thus, it is possible that environmental cues, such as high S1P levels in the blood and lymph, may cause epigenetic alterations that compensate for the SphK deficiency. In fact, we recently found that a passive cutaneous anaphylactic (PCA) challenge of SphK2-deficient mice results in a defective response (unpublished observation). Thus, this skin-localized challenge showed the expected impaired mast cell degranulation of the fetal liver-derived SphK2-deficient mast cells. This is consistent with low levels of S1P in the skin and the findings clearly demonstrate that the high levels of circulating S1P in these mice is a key factor in shifting the poor-responding phenotype of SphK2-deficient mast cells to one of normal response. Obviously, the function of the SphK isoforms is more likely to be unambiguously elucidated by tissue-specific gene targeting in adult mice.

Regulation of Mast Cell Calcium Responses by SphKs

Despite these unresolved issues and dissimilarities, overall the findings using pharmacological inhibitors, silencing RNAi approaches or genetic deletion models, all point to a critical role for SphKs in mast cell function and suggest that some potential overlap for the two isoforms might exist. What has not been studied in detail is the mechanism by which SphK1 or SphK2 affect mast cell function. Our studies on the fetal liver-derived mast cells from SphK2-deficient mice indicated that the underlying cause for the overall impaired mast cell responsiveness was due to an impairment of calcium influx and PKC activation.[31] In human mast cells, silencing RNAi approaches have implicated SphK1 in calcium release from intracellular stores.[39] However, we uncovered no evidence of a role for SphKs or S1P in mobilizing calcium from endoplasmic reticular (ER) stores. As described in another chapter in this book, calcium influx is well known to be an essential process for FcεRI-induced mast cell degranulation and cytokine production[48,49] and thus how SphK2 regulates calcium mobilization is of considerable interest. An essential characteristic of some calcium channels is their modulation by lipids[50] and S1P has been shown to regulate calcium mobilization.[6,51-53] SphK2-deficient mast cells fail to produce S1P and instead accumulate SPH, which has been reported to inhibit calcium influx.[54-57] In contrast, S1P added exogenously to BMMCs had no effect on either FcεRI-induced calcium mobilization[31] or store-operated calcium release-activated calcium current (iCRAC) in RBL-2H3,[55] suggesting that the effect of S1P on calcium is likely to be intracellular and not through binding of its cell surface receptors on mast cells. As alluded to above, work in RBL-2H3 cells has shown that SPH is an inhibitor of store-operated calcium release-activated calcium current (iCRAC)[55] and of the coupling of Orai1/CRACM1 to STIM1, the calcium sensor protein in the ER.[58] Since SphK2 controls the intracellular levels of S1P and SPH in mast cells in the resting and activated states, it is possible that an elevation in S1P directly or indirectly leads to the opening of a calcium channel, or that a reduction of SPH releases the inhibitory effect on calcium channel function.[31,55] In support of this view, Prieschl and colleagues[32] proposed a rheostat model based on the observations that addition of exogenous SPH inhibited mast cell degranulation and leukotriene and TNF production, effects that could be effectively reversed by S1P. The model stipulates that the balance between SPH and S1P is decisive for the excitability of mast cells. Apart from the possibility that S1P or SPH may directly modify the function of an ion channel in mast cells, a more indirect mechanism is also possible. Recent studies in our lab suggest that reduced levels of nonselective cation channel Transient Receptor Potential Channel 1 (TRPC1) mRNA and protein, in Fyn-deficient BMMCs, is a contributory factor for the defective calcium influx and degranulation of these cells (RS and JR, unpublished results). Moreover, silencing of TRPC1 expression in WT BMMCs also resulted in impaired calcium influx and defective degranulation. As previously mentioned, Fyn-deficient BMMCs failed to activate SphKs after FcεRI stimulation and we found that they also have lower baseline levels of S1P.[33] Since S1P was recently shown to affect nuclear transcription by acting directly on histone deacetylases[14] one possible scenario is that homeostatic S1P production may be involved in the regulation of certain genes critical for mast cell calcium responses, like TRPC1 and function. Thus, studies on S1P regulation of gene expression may shed further insights on the role of this lipid in regulating mast cell signals and function.

FcεRI INDUCES S1P FORMATION AND EXPORT

FcεRI-mediated activation of SphKs not only leads to the formation of S1P inside the cells,[37,39] but also results in its export into the extracellular space.[32,33,59] This suggests that the site(s) of action for S1P may be both intra- and extracellular. As previously mentioned, mast cells lacking SphK2, which do not produce intracellular S1P or export it, have a defect in calcium mobilization and degranulation and these defects are either not restored or only partially restored, respectively, when S1P is added exogenously. This suggests that the some actions of S1P (such as the effect on calcium influx) are independent of its cell surface receptors on the mast cell.[31] However, the function of S1P as a second messenger is not well understood and, other than the recent finding of its binding to and regulating histone deacetylase activity,[14] its intracellular targets are not defined. In contrast, it has been shown that the receptors for S1P participate in mast cell responses.[59] In many cell types, the export of newly synthesized S1P may occur as a localized process with coordinated S1P production on the inner membrane leaflet resulting in receptor activation on the outer leaflet, although the amounts of S1P generated are usually not readily detectable.[6,60] Notably, mast cells and platelets can release copious amounts of S1P upon agonist stimulation[8,20] suggesting that the S1P released plays a role beyond the autocrine modulation of cell function and may be an important paracrine component of mast cell and platelet regulation of immune responses.

Mechanisms of Export

Both platelets and mast cells secrete their granular contents by exocytosis. However, in mast cells, S1P is not pre-accumulated in granules and degranulation appears to be dissociated from S1P export.[61,62] Since S1P is a charged molecule, spontaneous flip-flop of S1P across the bilayer is not likely to occur and thus a facilitating transport mechanism must be in place. The ATP binding cassette (ABC) superfamily of transporters, which catalyze the transport of chemically diverse compounds across cellular membranes, have been involved in the transport of S1P, both from the outside to inside cells[63] or from the inside to the outside of the cell.[61,62,64] The ABCC7 ion channel CFTR was implicated in the take up of S1P from the mileu.[63] However, little is know about the role of this transporter in the uptake of S1P for mast cells. In contrast, several types of ABC transporters have been implicated in the efflux of S1P including ABCB1 in T cells,[64] an ABCA-like transporter in platelets,[62] ABCC1 in the RBL-2H3 and human LAD2 mast cell lines,[61] and ABCC1 and ABCG2 transporters in breast cancer cells.[65] Inhibition of ABCC1 or RNAi silencing of its expression efficiently reduced the FcεRI-stimulated export of S1P from mast cells, but it only partially reduced the constitutive export of S1P observed when SPH was added to the cells, suggesting other transporters or additional mechanisms of export might be present.[61] Interestingly, mast cells derived from S1P Lyase-deficient mice, which have markedly elevated levels of S1P and enhanced constitutive as well as FcεRI-induced efflux of S1P into the media, showed an upregulation of an ABCA1 transporter (L. Wright, Y. Kitamura, AO and JR, unpublished observations), although its relevance to S1P export in these cells is still unknown. Recently, it was shown[66] that in zebrafish, the protein Spns2, with structural homology to the bacterial glycerol-3-phosphate transporter, can specifically export S1P. A mutation in the *Spns2* gene resulted in the inability of yolk syncytial cells to release S1P during heart development, a critical event for the chemoattraction of myocardial precursors bearing the S1P receptor S1PR2 to

form the heart tube. A mutation in either *Spns2* or in the *S1PR2* genes led to the presence of a split heart; a phenotype that was rescued by injecting S1P, *Spn2 mRNA* or human *Spns2 mRNA*, but not the homolog *Spns1 mRNA*. Whether this type of transporter, or its homolog, may also function in mast cells has not been investigated. Mice carrying genetic deletions in ABC transporters or Spns2 may be pivotal to unequivocally define the type of transporter involved in the export of S1P and the contributory role of secreted S1P in allergy and inflammation.

FUNCTIONS OF S1P RECEPTORS IN MAST CELL RESPONSES

Mast cells have been described to express two types of receptors for S1P, S1PR1 and S1PR2,[59] although recent observations in our lab identified the presence of mRNA for S1P4 as well, but not S1PR3 or S1PR5. FcεRI activation of SphKs and the production of S1P was shown to induce a ligand-dependent transactivation of S1PR1 and 2 enhancing mast cell functions.[59] The transactivation of S1PR1 and S1PR2 can be readily recognized, as these receptors are endocytosed with β-arrestin, a common phenomena observed when a GPCR is activated.[67] Antisense oligonucleotide-mediated silencing of S1PR1 or S1PR2 indicated that while S1PR1 was involved in the migration of mast cells toward low concentrations of antigen, S1PR2 participated in FcεRI-induced degranulation. Silencing of S1PR1 reduced and ectopic expression of S1P1 enhanced, respectively, chemotaxis of mast cells towards antigen without affecting degranulation. On the other hand, overexpression of S1P2 inhibited the chemotactic motility of RBL cells towards antigen.[59] Moreover, S1PR2 mRNA expression, but not S1PR1 mRNA, was increased as a late consequence of FcεRI stimulation. These findings argue for a model whereby S1PR1 participates in the recruitment of mast cells to their site of action, while S1PR2 deters migration and contributes to degranulation once mast cells reached their site of action. Additional findings reinforce the conclusion that SphK activation, S1P generation and export and S1PR1 engagement is necessary for mast cell chemotaxis. Fyn-deficient mast cells, which fail to activate SphKs, also have defective chemotaxis in response to antigen and SCF, but exogenous S1P in combination with FcεRI-generated signals can partly restore the motility defect.[33] Similarly, pharmacological inhibition or genetic deletion of SphK in mast cells resulted in reduced chemotactic motility towards antigen or SCF, but these cells were able to migrate normally towards S1P[33,59] (AO and JR, unpublished results). Interestingly, inhibition of ABCC1-mediated S1P export blocked migration of mast cells to antigen in vitro and this was rescued by addition of S1P,[61] suggesting that mast cell chemotaxis to antigen or SCF is, at least in part, dependent on mast cell derived S1P.

The involvement of S1PR2 in degranulation is not as clear. Exogenous addition of S1P alone or in combination with FcεRI stimulation does not induce significant degranulation in the RBL-2H3 cell line or in murine mast cells.[33,59] However, RNAi silencing of S1PR2 or its genetic deletion in BMMCs resulted in a 40 to 50% reduction in FcεRI-induced degranulation. Of note, the degranulation response to ATP[59] and to phorbol esters and ionomycin (AO and JR, unpublished results) were also reduced in S1PR2-deficient BMMCs, suggesting a potential defect in the exocytotic machinery in these cells. Interestingly, inhibition of ABCC1-mediated S1P export did not affect FcεRI-induced degranulation, indicating that export of S1P by this transporter is needed for migration but not for degranulation.[61] Although it is possible that a yet unidentified transporter brings a distinct pool of S1P to the proximity of S1PR2, other possibilities include that the S1P/S1PR2

axis functions independently of a transporter for the degranulation response, or perhaps in a model similar to that proposed for other S1PRs[68] in which a constitutively active S1PR2 facilitates a signaling platform that promotes FcεRI signals. In this later model, the S1PR2 could act as a sensor for S1P in the environment and thus as a modifier of FcεRI responses. For example, in normal tissues where the levels of S1P are low S1PR2 activity on mast cells could also be low. Thus, in tissues, mast cells might require higher amounts of antigen to trigger a response, whereas in mast cells that are in closer vicinity to the circulatory system (where the S1P concentration is high or under conditions where S1P is elevated) the activity of the S1PR2 would be higher, lowering the threshold of activation via the FcεRI. Nevertheless, this is hypothetical and more conclusive evidence for the role of this receptor in mast cells responses in vivo is needed. Our recent studies of systemic anaphylaxis in the S1PR2-null mice suggest that these mice have a normal immediate hypersensitivity response upon systemic antigen challenge. However, we uncovered a mast-cell independent contribution of this receptor in the recovery from anaphylaxis and thus a mast-cell specific genetic deletion of S1PR2 is needed to more directly assess its involvement in the mast cell responses during anaphylaxis or other allergic disorders.

Paracrine Function of Mast Cell-Released S1P

As tissue-resident cells, mast cells are exposed to a variety of stimuli whose profile can change given a particular circumstance. Antigen-stimulation of FcεRI, for instance, has been demonstrated to induce the production and release of S1P into the interstitium and thus S1P may participate in promoting allergic inflammation. It is known that the levels of S1P also increase locally during acute inflammation in the footpad,[18] in the airways of asthmatic individuals hours after a challenge,[69] and in the joints of rheumatic patients.[70,71] The origin of the elevated S1P in tissues under these conditions is not known, but one reasonable candidate is the mast cell. Changes in S1P levels can have drastic effects in the surrounding tissue, not only by affecting angiogenesis and vascular permeability,[1,72] but also by causing fundamental changes to the immune system.[7,70,73] However, the specific role of S1P in inflammation or in allergic inflammation is still unclear, but it is likely that the particular challenge and the type of immune cells that respond to this challenge may be a determinant of S1P function in inflammation. Increases in the levels of S1P and/or increased expression of S1PRs can result in the accumulation of immune cells in the local site. Several mechanisms may account for this accumulation: induction of chemotaxis,[29,59,74-78] retention of hematopoietic stem cell progenitors,[19] and retention of mature T cells in the tissues; the latter two through increased LFA-1–ICAM-1 and VLA-4–VCAM-1-mediated adhesion to the afferent lymphatic endothelium.[18] In vitro and in vivo experiments in murine systems suggest that S1P can also cause a shift in T-cell responses promoting T_H2- and T_H17-cell responses while disfavoring T_H1-cell responses, thus potentially skewing responses toward allergic phenotypes. This, in part, can be mediated through the reported effects of S1P on dendritic cell maturation. S1P was shown to impair the ability of lipopolysaccharide (LPS)-stimulated human monocyte-derived DCs to cause naïve $CD4^+$ T-cells differentiation to T helper 1 (T_H1)-cells and instead promoted T_H2-cell differentiation.[7,76] In this regard, it should also be kept in mind that S1P also seems to be important in the effector phase of allergic disease through control mast-cell responsiveness. Thus, in certain circumstances, it appears that S1P can play a major role in promoting allergic inflammation.

An anti-inflammatory role for S1P has also been described. For example, S1P can induce a switch from proinflammatory M1 to the anti-inflammatory M2 macrophage subtype,[7] which explains the observed protective effect of S1P against atherosclerotic lesions.[79] Furthermore, topical application in the skin[80] or inhalation of S1P or the S1P mimetic FTY720[81] suppressed, respectively, ear swelling in a model of allergic contact dermatitis and airway inflammation and bronchial hyperresponsiveness in an asthma model. The underlying mechanism for suppression appears to be the inhibition of dendritic or Langerhans cell migration to the lymph nodes and thus an impaired generation of allergen-specific T cells.[80,81] Furthermore, FTY720 also induced a decrease in the induction of T_H1- and T_H2-cell effector cytokines and the establishment of a stable synapse between DCs and T cells.[81] Intriguingly, lesional skin biopsies of individuals with atopic dermatitis[82] and psoriasis[83] show increased expression of two enzymes involved in the degradation of S1P, S1P Lyase and S1P-phosphatase Type 2 (SPP2), respectively. The elevated presence of these enzymes could result in reduced levels of S1P and the perpetuation of the disease. Consistent with this view, an anti-inflammatory role for S1P was also suggested by the reduction in IL-1β and IL8 production by stimulated HUVEC or neutrophils when its degradation was blocked by RNAi silencing of SPP2.[83] Alternatively, the SPP2-mediated conversion of S1P to SPH, which can be used to generate ceramide,[17] or the breakdown of S1P by S1P-Lyase into metabolites that can be used for the synthesis of other lipids, particularly phosphatidylethanolamine,[16] may also play a role in the course of these distinct diseases by affecting the lipid composition of the skin. Interestingly, application of an emulsion of ceramide to the skin appears to reduce the severity of atopic dermatitis.[84-86] However, at the moment it is difficult to distinguish if the increase in expression of enzymes involved in the catabolism of S1P contributes to the development of the disease by reducing the presence of an anti-inflammatory S1P, or if this is a mechanism to suppress disease by eliminating a pro-inflammatory S1P and producing other lipid products that improve the symptoms.

S1P-MEDIATED REGULATION OF THE MAST CELL PHENOTYPE AND RESPONSIVENESS

Increased levels of S1P in the circulation were found to correlate with the ability to release granule-stored mast-cell allergic mediators (degranulation), such as histamine. This was unexpectedly uncovered when we compared the responses of mast cells derived from SphK-deficient mice in vitro to the responses of mast cells during IgE-dependent systemic anaphylaxis. SphK-deficient mice have altered levels of circulating S1P and these changes in S1P levels are independent of the mast cells. [31] Genetic deletion of SphK1 resulted in the reduction of circulating S1P,[31,46] because this isozyme is the major activity present in RBC, the cells most responsible for maintaining S1P levels in the blood. In contrast, genetic deletion of SphK2 caused an elevation in the circulating levels of S1P,[31,47,87] perhaps due to a compensatory elevation of SphK1 activity in RBC. As previously mentioned, FcεRI-dependent stimulation of SphK2-deficient mast cells in vitro resulted in a defective degranulation response. However, when SphK2-deficient mice were challenged systemically, in an IgE/Ag-dependent manner, the mast cell response (as measured by histamine release) was similar to WT mice suggesting that the higher levels of S1P in these mice could cause SphK2-deficient mast cells to function normally. In contrast, SphK1-deficient mice, whose mast cells showed normal degranulation in vitro,

had decreased histamine release upon a systemic challenge. This apparent disconnect between the in vitro and in vivo results appeared to indicate that the exposure to high or low levels of S1P caused an alteration of the mast-cell phenotype[31] (Fig. 2 and Table 1). As discussed earlier, it was also possible that the phenotype of the mast cells differentiated in vitro did not reflect the in vivo phenotype of mast cells participating in the anaphylactic response. However, additional experiments (AO and JR, unpublished observations) showed that a local (cutaneous) IgE/Ag-challenge of SphK1- or SphK2-deficient mice showed a normal or defective mast cell response, respectively, that mirrored the results observed

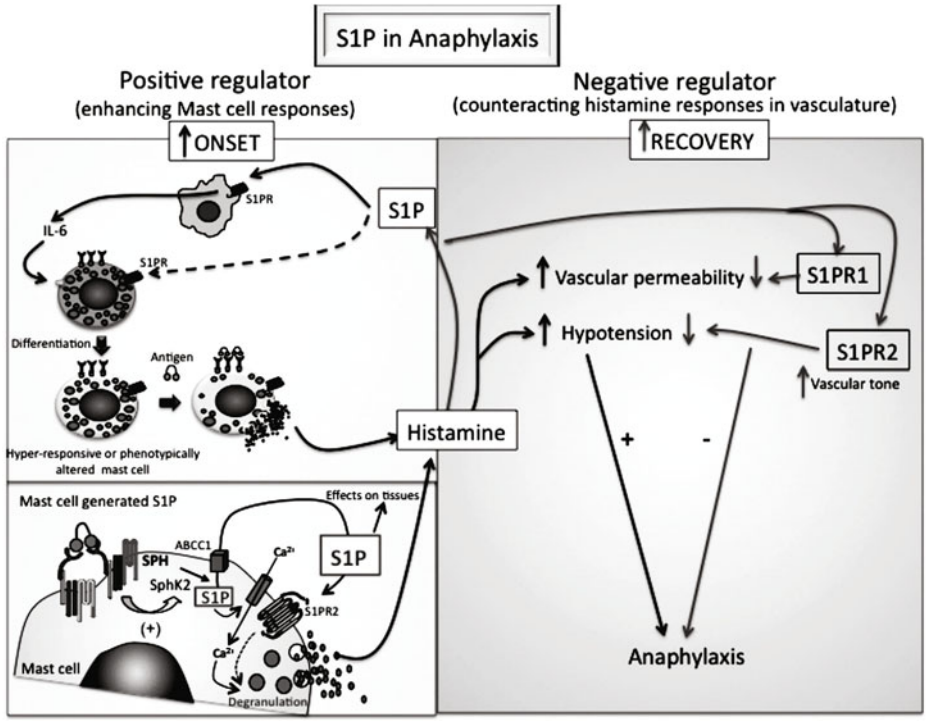

Figure 2. Dual role of sphingosine-1-phosphate in the regulation of systemic anaphylaxis. While S1P can enhance the signaling and function of mast cells (left panels), which initiate the allergic response, it alleviates the symptoms of the anaphylactic shock acting in a mast cell-independent manner (right panel). Exposure of mast cell progenitors to higher levels of S1P in the cellular environment can change the differentiated phenotype and/or enhance the responsiveness of mast cells. The effect of S1P on mast cells can be indirect, involving monocytes/macrophages, or direct (left, upper panel). Thus, under circumstances where S1P is increased, mast cells can become more sensitive to a stimulus and thus influence the initiation of an allergic response. Mast cells can produce S1P via the activation of SphKs (left lower panel). The activation of SphK2 by FcεRI is necessary for the activation of calcium channels at the plasma membrane and for the degranulation of mast cells. The S1P generated is exported to the outside of the cell, where its concentration increases locally and can thus engage the S1PRs on the mast cells or on other neighboring cells, affecting the inflammatory response. The release of histamine by mast cells triggers SphK1 activity and the production of S1P from nonmast cell sources (right panel). This S1P activates S1PR2 receptors in the vasculature, regulating the vascular tone and thus blood pressure, which in turn can control the glomerular filtration rate and the clearance of histamine, resulting in the recovery from anaphylaxis. SphK1 and S1P production can also activate S1PR1 receptors, which prevent vascular leakage and this may also contribute to recovery.

in vitro (Table 1). This was not due to a difference in the number of mast cells in the skin or differences in granule content since the local (cutaneous) response to compound 48/80, a secretagogue, was comparable in all the mutant and WT mice. This suggests that, in systemic anaphylaxis, changes in the levels of circulating S1P may influence the responsiveness of the mast cell and thus the sensitivity and/or severity of this response. In the skin, where levels of S1P are inherently low, the role of mast-cell extrinsic S1P on mast cell responsiveness may not be so influential.

Additional data in models other than SphK-null mice also support the idea that in vivo regulation of S1P homeostasis (extrinsic to the mast cell) can regulate mast cell responsiveness. Mice from an 129Sv genetic background, which undergo strong T_H2-cell responses and anaphylaxis,[88] have higher levels of circulating S1P as compared to C57BL/6 mice, which have modest T_H2-cell responses.[89] Increasing the levels of circulating S1P in C57BL/6 mice, by using an inhibitor of S1P lyase,[15] results in mice with enhanced mast cell responses as determined by increased histamine in the plasma following a systemic challenge. The level of the response resembles that observed in 129Sv mice, suggesting a link between S1P levels and the enhanced mast cell phenotype (AO and JR, unpublished observation). Recent findings also indicate that exposure of human cord blood progenitors to both S1P and stem cell factor enhances the rate of differentiation of these progenitors to mast cells and causes them to become more like a connective tissue type mast cell (containing chymase and tryptase in their granules) rather than a mucosal type (containing tryptase) mast cells, which is the normal when cells are cultured in the presence of stem cell factor alone.[35] The mechanism for how S1P causes the developmental shift in the phenotype of human mast cells appears to be partially indirect, involving the production of IL-6 by cells of monocyte/macrophage lineage.[35] Our unpublished observations on the effect of S1P during murine BMMCs differentiation indicate that chronic treatment with S1P induces genetic changes that give rise to a hyper-responsive mast cell. This may be a direct action of S1P on the mast cells, but whether this is mediated by a S1PR on the mast cell remains to be determined.

Thus, S1P homeostasis in circulation or the tissue environment, which can be dysregulated by genetic causes or by dietary or environmental factors, may result in alterations in the phenotype of mast cells and the susceptibility to particular allergic reactions. Although the discussed data supports an effect of the extrinsic S1P in vivo as a regulator of mast cell responsiveness, other findings argue otherwise. Adult mice lacking S1P, by conditional deletion of both SphK1 and SphK2, had normal mast cell responses to an anaphylactic challenge when challenged with strong stimuli,[23] suggesting normal mast cell function in the absence of S1P. Additional studies are needed to determine if the susceptibility of mast cells to S1P in the environment or within the mast cell depends on the type or the strength of the stimulus.

RECOVERY FROM ANAPHYLAXIS IS POSITIVELY REGULATED BY S1P FROM A NON-MAST CELL SOURCE

S1P receptors are ubiquitously expressed and affect multiple physiological functions in various tissues.[7] Thus, it is likely that, beyond their role in mast cell biology and function, these receptors may influence other aspects of the allergic disease. For instance, S1PR1, S1PR2 and S1PR3, are known to regulate the vascular system affecting vascular tone, vascular permeability and heart rate.[90-92] In endothelial cells, S1PR1 preserves vascular

integrity and regulates vascular permeability.[23,91,93] S1PR3 functions in myocardial contractility and in the regulation of bradycardia and hypertension induced by S1P;[94] and S1PR2 increases vascular permeability and regulates renal, mesenteric and local blood flow in various organs.[91,95,96] All these processes are vastly altered during an allergic reaction like systemic anaphylaxis. Thus, it is not surprising that S1P produced during anaphylaxis by mast cells, or other cells, could influence the severity of the response by acting on the vascular system. Recently, we discovered[9] that S1PR2 plays an important role in counteracting the vasodilator actions of histamine and therefore the hypotension associated with systemic anaphylaxis (Fig. 2). We found that histamine drives S1P production by activating SphK1 and this is required for the engagement of S1PR2 and the recovery from anaphylaxis. Genetic deletion of SphK1 or S1PR2 disrupts this regulatory loop during histamine or IgE/Ag-induced anaphylaxis, causing a severe hypotension, with a consequent delay in the renal rate of histamine excretion and thus a delay in the recovery from anaphylaxis.[9] Importantly, the delayed recovery from anaphylaxis could be rescued in SphK1-deficient by intravenous injection of S1P during anaphylaxis. However, S1P injection had no effect on the delayed recovery of S1PR2-deficient mice, indicating that the presence of this receptor is required for S1P-induced rescue from anaphylaxis.

Other studies suggest that S1PR1 may also play a role in recovery from anaphylaxis.[23] Inducible genetic deletion of both SphK1 and SphK2 results in mice that lack circulating or tissue S1P. When challenged with IgE/Ag or PAF, these mice undergo a severe anaphylactic response with resulting lethality. The IgE (SPE-7 clone) and PAF used in this study are potent inducers of mast cell degranulation. A massive loss of circulatory fluids by vascular extravasation was observed and this was attributed to the loss of S1PR1 function, since lethality was partially prevented by injection of a S1PR1 agonist.[23] Similar anaphylactic lethality was also seen when SphK1-deficient mice were challenged with PAF and these mice also showed enhanced vascular fluid extravasation.[23] In contrast, we did not observe differences in vascular fluid extravasation between WT and SphK1-deficient mice in our model of IgE/Ag (using a H1-DNPε clone) or histamine-induced anaphylaxis, although SphK1-deficient mice clearly showed more severe anaphylaxis. Thus, whether S1PR1 or S1PR2 is dominant in recovery from anaphylaxis may well depend on the stimuli. Alternatively, models where lethality is the measureable outcome may not reflect the normal anaphylactic response to a stimulus, since recovery is common.[97] Regardless, these two different studies suggest that generation of S1P during anaphylaxis and/or the presence of S1P in the circulation have a protective effect via the activation of either or both S1PR1 and S1PR2. While S1PR1 plays a role in the maintenance of the vascular barrier integrity, S1PR2 regulates the vascular contractility that allows for the recovery from hypotension resulting from a systemic anaphylactic challenge. It is known that, in humans, the two main anaphylactic triggers are IgE- or IgG-mediated and the type and the prevalence of released mediators differs, with PAF release being more abundant in IgG-mediated anaphylaxis. Thus, from a therapeutic perspective, a combination of S1PR1 or S1PR2 agonists used at different ratios may prove useful in the treatment of anaphylaxis. In this regard, we found that treatment of WT mice with S1P during histamine-induced anaphylaxis expedited their recovery from anaphylaxis at a similar rate as treatment with adrenaline, the first choice of treatment for anaphylaxis. However, administration of adrenaline carries some risks, particularly in the elderly population, including cardiac arrhythmias, myocardial infarction and hypertensive intracerebral bleeds.[98,99] Since the S1PR2 receptor does not mediate bradycardia, tachycardia or

influence myocardial function in vivo and its effects on blood pressure and flow rate appear to be mostly vascular,[95] specific receptor agonists may be a safer and useful alternative for anaphylaxis treatment. As a note of caution, it is important to point out that S1PR2 has the opposite role of S1PR1 in vascular permeability, as its activation in the endothelial cells can lead to increased vascular permeability. However, the fact that the S1PR1 is predominantly expressed in endothelial cells and that S1PR2-deficiency in our model of histamine-induced anaphylaxis did not reveal differences in vascular permeability, provides an argument for further exploration of S1PR2 agonists in counteracting anaphylaxis.

Overall, another important conclusion suggested from the studies on anaphylaxis is that SphK/S1P is a "Janus" regulator of the systemic allergic response (Fig. 2). On one hand, it is part of the signals generated upon engagement of FcεRI and functions to enhance the activation of mast cells. Also, the presence of S1P in the microenvironment may render mast cells with a more responsive phenotype. On the other hand, SphK/S1P also seems to regulate other aspects of the allergic response that are independent of the mast cell.[9] In the case of systemic anaphylaxis, although it enhances mast cell immediate responses, it also mediates the recovery from shock, thus affecting positively and negatively, respectively, the onset and duration of anaphylaxis. Such dual roles for physiological mediators are not unusual in biology since, in a similar way, a given cytokine may have distinct roles as an initiator or attenuator of the immune responses.[100-102]

CONCLUSION

SphK1, SphK2, their product S1P and the receptors for S1P are all emerging as important regulators of allergic responses. Their roles extend beyond the regulation of the effector function of mast cells and other immune cells to the regulation of the surrounding environment and the (patho)physiological response. Due to the complexity of their actions and the various types of cells involved, further investigations must be aimed at determining the specific sites of action and the relative importance of these molecules to the resulting outcomes. The models that completely eliminate the expression of these molecules (through genetic deletion) in the entire mouse have been useful so far, but they are limited in allowing a further understanding of the role of S1P in different cell types and compensatory mechanisms may complicate the interpretation of their observed involvement. For example, studies on the development of arthritis in SphK1-deficient mice indicated that SphK1 had no involvement in the severity and incidence of inflammation.[103] However, studies where pharmacological inhibitors of SphK or RNAi for SphK1 were administered, showed that the incidence, severity and articular inflammation were reduced.[71,104] Although the reasons for these discrepancies are not entirely clear, one of the possibilities is that the pharmacological inhibitors or the silencing RNAi may target a population(s) of cells that is more susceptible to these treatments and are essential in the disease progression. Tissue-specific targeting of the SphKs or S1PRs for deletion in specific cells would be highly beneficial in evaluating these possibilities. This could be particularly valuable when considering SphKs or S1PR2 as targets for therapy and in furthering our understanding of their role in health and disease. Nevertheless, we conclude from the work cited herein that SphKs, S1P and S1PRs are key components in the regulation of allergic responses and beyond.

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CHAPTER 9

DOWN-REGULATION OF MAST CELL RESPONSES THROUGH ITIM CONTAINING INHIBITORY RECEPTORS

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Abstract: The multiple cell types that comprise the immune system provide an efficient defense system against invading pathogens and micro-organisms. In general, immune cells are activated for disparate functions, such as proliferation, production and release of mediators and chemotaxis, as a result of interactions between ligands and their matching immunoreceptors. This in turn leads to the recruitment and activation of a cascade of second messengers, via their regulators/adaptors, that determine the net effect of the initial response. However, activation of cells of the immune system must be tightly regulated by a finely tuned interplay between activation and inhibition to avoid excessive or inappropriate responsiveness and to maintain homeostasis. Loss of inhibitory signals may disrupt this balance, leading to various pathological processes such as allergic and auto-immune diseases. In this chapter, we will discuss down-regulating mechanisms of mast cells focusing on immunoreceptor tyrosine-based inhibition motifs (ITIM)-containing inhibitory receptors (IR).

INTRODUCTION

Mast cells are the main effector cells of allergic reactions as well as are important regulators of a number of pathophysiological processes. Mast cells can be activated by the binding of ligands to various receptors expressed on the cells. Nevertheless, the main mechanism of mast cell activation in allergy is through the binding of an allergen to the FcεRI-IgE complex. Positive or activation signals, which are transmitted through immunoreceptor tyrosine-based activation motifs (ITAM)-bearing receptors,

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are counterbalanced by negative or inhibition signals transmitted by immunoreceptor tyrosine-based inhibition motifs (ITIM). This is especially important to maintain the balance between activation and inhibition and to avoid hyperresponsiveness. In this chapter we will discuss in depth the down-regulation of IgE-mediated mast cell responses through ITIM-mediated inhibition signals.

CELL ACTIVATION

Cells of the immune system can be activated through a wide array of receptors, such as receptors for the Fc portion of different immunoglobulin subclasses (FcR), cytokines receptors, T-cell antigen receptor (TCR), the B-cell antigen receptor (BCR), Toll-like receptors (TLR), integrin receptors and G protein coupled receptors including those for specific chemokines, complement components, prostaglandin E_2 and adenosine. Ligands for the aforementioned receptors mediate their effects on cells through the activation of receptor-linked signal transduction pathways.

A pivotal mechanism of cell activation is carried out by Fc receptors. The extracellular domains of these receptors determine the specificity of the response and the types of cells involved. On the other hand, the intracellular domains of the Fc receptors are crucial for transducing signals into the cells and eventually for determining the duration and intensity of the initiated response.[1] The cytoplasmic portion of the Fc receptors contains immunoreceptor tyrosine-based activation motifs (ITAMs) through which the immune system transduces positive signals. Upon ligand recognition and receptor co-aggregation, tyrosine residues in the ITAMs are phosphorylated by Src family protein tyrosine kinase(s) (PTK(s)). The phosphorylated ITAMs serve as docking sites for the SH2 domains of Syk family PTKs, such as ZAP-70 or Syk. Syk family PTKs phosphorylate a series of substrates, leading to the formation of membrane-proximal scaffolds. In turn, important effector molecules, such as phospholipase C (PLC), are recruited leading to calcium signaling, as well as Ras activation, resulting in stimulation of the ERK pathway and cellular activation.[2]

In this chapter we will focus primarily on the IgE-mediated mast cell activation mechanism. The heterotetrameric high-affinity IgE receptor (FcεRI) on mast cells consists of three subunits: α, β and γ. The α subunit is unique to this receptor while the γ subunit is common to other receptors such as FcγRIIa. The β chain is important for stabilizing the receptor and for amplifying its signaling. Upon cross-linking of IgE bound to the FcεRI receptor with the multivalent antigen, FcεRI complexes aggregate and lead to a transphosphorylation of the ITAMs situated in the cytoplasmic regions of the β and γ chains. Tyrosine residues in the ITAMs are then phosphorylated by the src family kinase Lyn to initiate the signaling cascade[13] as above.

CELL INHIBITION/TERMINATION OF IMMUNE RESPONSES

As mentioned, immune responses are critical to fight pathogens and one of the main responses normally carried out is the classical inflammatory response. However, once the pathogen is removed, the inflammatory reaction must come to an end to avoid a chronic process. It is for this reason that multiple mechanisms exist to terminate or to down-regulate the activation and the recruitment of inflammatory cells and hence the inflammatory response. Activation of the immune system leads to the production and

Table 1. Human and murine inhibitory receptors expressed on mast cells

Subfamily	Human	Murine Counterpart	Ligand	Phosphatase Recruited	Inhibitory Component	Additional Cells Where Receptor Is Expressed
Ig-super-family	FcγRIIB	FcγRIIB	Immune complex-es lgG Abs	SHIP	1 ITIM	B cells
	Siglec-8	-	6′sulfo-sLex	SHP-1	1 ITIM; 1 ITIM-like	Eosinophils
	CD300a	Lmir-1	Unknown	SHIP; SHPs	4 ITIMs	Eosinophils
	SIRP-α	-	CD47	SHP-1,2	2 ITIMs	Myeloid, hematopoetic neu-rons
	(LILRB4)*	Gp49B1	αvβ3	SHP-1,2	4 ITIMs	Neutrophils, Macrophages, NK
	ILT/CD85	PIR-B	MHC-1 molecules	SHP-1 (SHP-2)	4 ITIMs	B cells, DC, Monocytes, Macrophages
	LAIR-1	LAIR-1	Collagens	SHPs	2 ITIMs	Most cells of the immune system
	PECAM-1	PECAM-1	CD38, αvβ3	SHP-2, SHP-1	2 ITIMs	Endothelial, Neutrophils, NK, Monocytes, Platelets
	CD200R	CD200R	CD200	SHIP	NPXY motif	Basophils
C-type lectin like	MAFA	-	E-cadherin	SHIP, SHP-2	1 ITIM	NK, Basophils, U937 (human) NK, CD8-T cells (mouse)
	CD72	-	CD 100	SHP-1	2 ITIMs	B-cell, basophils

*Receptor was not found to be expressed on mature human mast cells at the protein level.[49]

secretion of arachidonic acid metabolites (i.e., prostaglandins, thromboxanes, leukotrienes) and of pro-inflammatory cytokines such as IL-8, IL-1 and TNF-α. Moreover, other specific mediators produced during inflammation include histamine from mast cells, elastase from neutrophils and perforin from NK cells. Therefore, one way to end the inflammatory response is to stop the production and/or secretion of such mediators. Indeed, a natural mechanism of self "extinguishing" the acute phase response occurs due to the relatively short half-lives of the lipid metabolites.[3] Similarly, production of anti-inflammatory cytokines (i.e., IL-10, TGF-β) and down-regulation and desensitization of immunoreceptors are also crucial for the termination of inflammation.

Apoptosis or programmed cell death can also end inflammation.[4,5] This can occur through two main signaling pathways: an intrinsic one mediated by the mitochondria; and an extrinsic one mediated by death receptors. Both pathways rely on a family of intracellular cysteine proteases called caspases.[6] The expression and function of two death receptors, FAS/CD95R and TRAIL-R, has been shown in both murine and human mast cells.[7] An additional mechanism to end an inflammatory process is through the resolution or catabasis process. This is an active process that leads to the reduction or removal of leukocytes and debris from inflamed sites. The resolution stage is initiated after an acute challenge by cellular pathways leading to the biosynthesis of anti-inflammatory, pro-resolution lipid mediators such as lipoxins, resolvins and protectins.[8]

In addition to the mechanisms mentioned above regulating immune responses, there has been a growing interest in a family of receptors that mediate inhibitory responses and are therefore named "inhibitory receptors". As opposed to the positive signals transmitted through ITAM-bearing receptors, negative regulation of the immune system is controlled by immunoreceptor tyrosine-based inhibition motifs (ITIM).

ACTIVATION AND DOWN-REGULATION OF MAST CELL RESPONSES: GENERAL

Mast cells are highly granulated, FcεRI-bearing tissue-dwelling cells that develop from myeloid progenitors expressing CD34, CD117 (Kit) and CD13, under the influence of stem cell factor (SCF). Mast cells have a "lead" role in allergic reactions, but are also involved in regulating fibrosis, in tissue responses to neoplastic diseases, in autoimmune diseases and in host-defense against bacterial and parasitic infections[9] (also reviewed in other chapters). By their activation and consequent release of mediators, mast cells cause the early symptoms of allergy and orchestrate and actively participate in the later chronic stages by releasing chemotactic factors for blood-borne inflammatory cells and by producing growth factors and activators for the same cells infiltrated in the tissues. Mast cells can be activated by various stimuli in addition to "the classical" binding of an allergen to the FcεRI-IgE complex. For example, SCFwhose receptor Kit is expressed on mast cells can induce cell differentiation, maturation, chemotaxis and survival; and also potentiate antigen-mediated mast cell activation.[10] Other examples of mast cell activation are through the binding of a ligand to other receptors such as: TLRs; and cytokine, chemokine, complement and neuropeptide receptors, all of which are also expressed on mast cells.[11,12] Moreover, basic or polybasic substances such as some endogenous mediators (i.e., substance P, bradykinin, neurotensin, eosinophil derived major basic protein, etc.) or drugs (i.e., codeine, morphine, desferroxamine), or synthetic peptides (compound 48/80) can similarly stimulate mast cells.[10]

INHIBITORY RECEPTORS: ITIM, ITAMi AND ITSM

Inhibitory receptors were initially identified and characterized on NK cells, where their ligands are primarily MHC-I molecules. The interaction of MHC-I with inhibitory receptors on NK cells leads to the abrogation of signaling pathways and therefore prevents the cell's cytotoxic activity against self.[13] Inhibitory receptors have now been shown to be expressed on multiple cell types in the immune system, including T cells, B cells, mast cells, eosinophils and neutrophils (Table 1).

The inhibitory receptors belong either to the Ig-receptor super-family or to the c-type (calcium dependent) lectin super-family. As mentioned above, inhibitory receptors contain one or more ITIM sequences. Classical ITIMs are defined as a consensus of a six amino acid sequence, I/V/LxYxxL/V (x denoting any amino acid), present in the intracytoplasmic domain of transmembrane molecules with inhibitory properties which, when phosphorylated, recruit SH2 domain-containing cytosolic phosphatases.[14,15] The Ig-like super-family inhibitory receptors are characterized by the presence of a single V-type Ig like domain in the extracellular portion. This family includes FcγRIIB, sialic acid binding Ig-like lectins (siglecs), signal regulatory protein α (SIRPα), Ig-like transcripts/leukocyte immunoglobulin receptors (ILTs/LIRs), killer cell Ig-like receptors (KIR), platelet endothelial cell adhesion molecule (PECAM-1), carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1), leukocyte-associated Ig-like receptors-1 (LAIR-1), CMRF-35H and paired Ig-like Type 2 receptor α (PILR-A). Inhibitory receptors that belong to the C-type lectin superfamily derive their name from the homology in their extracellular domain to the C-type lectins. These are known to be calcium-dependent lectins which bind carbohydrates through a specific domain named carbohydrate-recognition domain (CRD) (Table 1).[16,17]

As shown in figure 1, typically, ligand engagement by inhibitory receptors containing one or several ITIMs, suppresses cell activation by promoting dephosphorylation reactions. Upon ligation/activation of ITIM-containing receptors, tyrosine residues within these motifs become phosphorylated after the activation of receptor tyrosine kinases or Src family member tyrosine kinases (SFKs). This leads to the recruitment of the protein phosphatases, Src homology 2 domain-containing tyrosine phosphatase (SHP)-1 and SHP-2, or the lipid phosphatase, Src homology 2 domain-containing inositol 5- phosphatase (SHIP) 1. SHP-1/2 dephosphorylate tyrosine-containing signaling molecules and therefore counterbalance the action of tyrosine kinases. On the other hand, SHIP1 dephosphorylates phosphatidylinositol 3,4,5 trisphosphate at the 5′ position, thereby terminating PI3K-mediated signaling pathways.[12] The dephosphorylation of these signaling proteins leads to the dampening of cellular activation. It is important to note that the different ITIM-bearing inhibitory receptors mediate their actions through different effector phosphatases. This partially determines the net effect of the inhibitory response. Moreover, although inhibitory receptors are usually characterized by an ITIM in their cytoplasmic tails, some of the newly discovered inhibitory receptors contain either an ITIM-like sequence or other tyrosine-containing motifs that are crucial for the inhibitory functions of the receptor.

Apart from ITIMs, certain immunoreceptors contain immunoreceptor tyrosine-based switch motifs (ITSMs). Like ITIMs, ITSMs can associate with SHP-1/2 or with SHIP. ITSMs can also bind adaptor molecules such as SH2-domain-containing protein 1A (SH2D1A) and EWS-activated transcript 2 (EAT-2). Moreover, ITSMs can bind to Src family kinases and the p85 regulatory subunit of PI3K.[18] Therefore, ITSMs can

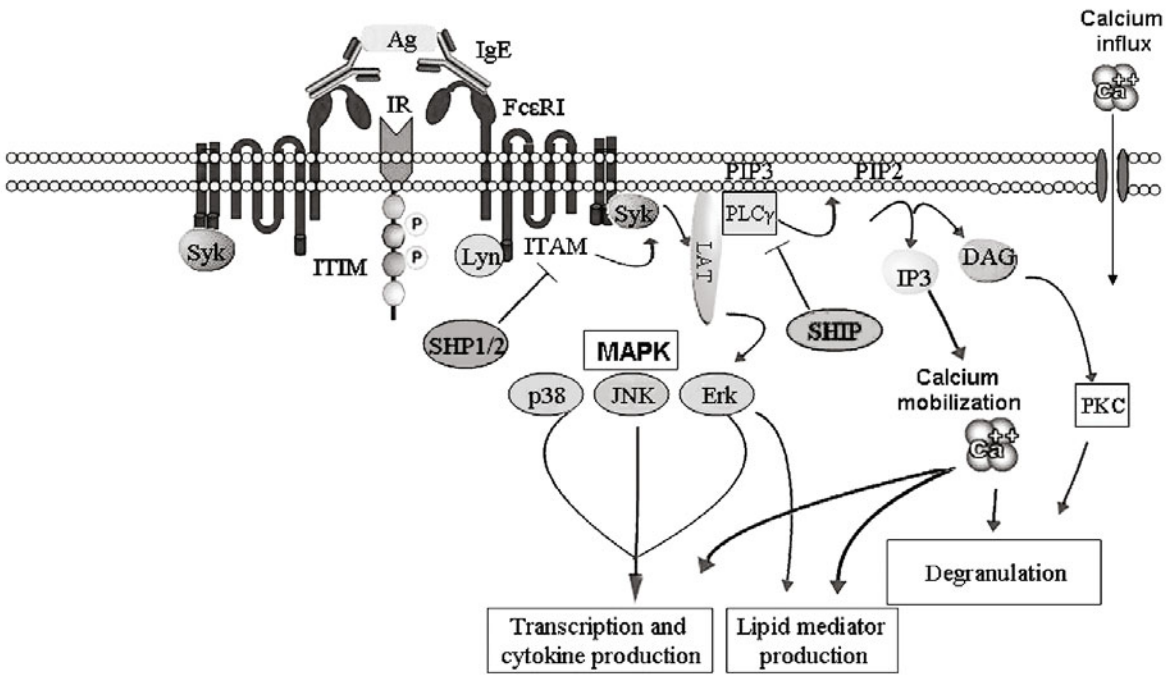

Figure 1. Schematic representation of ITIM-dependent inhibition of FcεRI-mediated activation. Mast cell FcεRI aggregation by receptor-bound IgE with an antigen leads to the phosphorylation of the receptors immunoreceptor tyrosine-based activation motifs (ITAMs). Tyrosine residues in the ITAMs are then phosphorylated by the kinase Lyn. Another tyrosine kinase (Syk) is also recruited to the complex and phosphorylates LAT. The latter activates PLCγ to degrade $PI(4,5)P_2$ to IP_3 and DAG. IP_3 induces intracellular Ca^{2+} mobilization followed by an extracellular influx. LAT also serves as a docking site for adaptor molecules leading to a downstream activation of MAPKs (JNK, p38, ERK). Subsequently, mast cell degranulation, cytokine and lipid mediator production occurs. Co-aggregation of an inhibitory receptor with FcεRI leads to the phosphorylation of the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the inhibitory receptor, leading to the recruitment of tyrosine phosphatases i.e., SHP-1/2 and SHIP. SHP-1/2 dephosphorylates the FcεRI β and γ chains as well as Syk. SHIP dephosphorylates PIP_3 to for PIP_2 thus abrogating downstream signaling and Ca^{2+} influx. Therefore, mast cell activation, i.e., cell degranulation as well as cytokine production, is abrogated. (The scheme was built using the "pathway builder" at www.proteinlounge.com).

transduce an activating or inhibitory signal depending on the immune receptor that they are associated with and the cell type in which they are expressed. The inhibitory receptor Lmir-3, which is expressed on murine mast cells and other myeloid cells, has been shown to impair mast cell cytokine production. Lmir-3 contains five tyrosine residues, two of which (Y241, Y289) are in the ITIM sequence, one in the ITSM and two (Y276, Y303) in the binding motifs for p85 or Grb2, respectively. The existence of the latter indicates a possible activating potential, in addition to its inhibitory one.

The 2B4 receptor, first described on NK cells, can also transduce activating and inhibitory signals. The inhibitory signals are mediated by the phosphorylation of its third ITSM and subsequent recruitment of SHP-1/2 and SHIP.[19] 2B4 has also been shown to be expressed (and functional) on murine mast cells.

In addition to the inhibitory ITIM mechanisms, it has been shown that under specific conditions, ITAMs can also transduce inhibitory signals. This led to the term inhibitory ITAMs or ITAMi. One of the known examples of such dual effects is mediated through immunoglobulin IgA and its receptor, FcαRI. Upon multimeric ligand binding and FcαRI aggregation, an inflammatory response is initiated. In contrast, following binding of a monomeric or low-valency ligand, with no sustained receptor aggregation, an inhibitory anti-inflammatory response is induced. Therefore, it seems that the type of interaction with the ligand determines the balance between inhibitory and activating functions of FcαRI. Moreover, ITAM-containing adaptors, FcRγ and DNAX-activating protein 12 (DAP12), have been shown to carry out dual functions. FcRγ has been shown to be involved in the inhibition of IgG-mediated phagocytosis in monocytes and IgE-mediated degranulation in mast cell transfectants.[20] Additionally, DAP12, associated with various immunoreceptors, mediated the inhibition of macrophages responses to pathogen-initiated signals through TLRs.[21]

Studies have demonstrated inhibition of activating responses of heterologous receptors such as FcγR or FcεRI, using anti-FcαRI Fab antibodies. These inhibitory effects are associated with the recruitment of SHP-1 as observed in ITIM-mediated inhibition, as well as a resultant decrease in FcεRI-induced ERK, LAT and Syk phosphorylation.[20]

In summary, although ITAM-activating responses are counter regulated by ITIM-regulated inhibition, ITAMi-mediated inhibition has emerged as a new aspect of negative regulation to cell activation. However, ITAMi inhibition appears to act as a more general mechanism in the long term maintenance of balance in the immune system. It is important to note that inhibitory receptors exist in human as well as murine cells, while some of the human receptors do not always have a murine counterpart.

MAST CELL-ASSOCIATED INHIBITORY RECEPTORS

The Ig-Like Superfamily

FcγRIIB

The FcγRIIB was the first inhibitory receptor in which the ITIM function was recognized, having been shown to inhibit B-cell receptor (BCR)-mediated activation in vitro.[22] It is a receptor for the immunoglobulin G constant (Fc) region and a Type-I transmembrane protein consisting of 291 amino acids containing one ITIM which has been shown to be expressed in both murine bone marrow derived mast cells (BMMC)

and human cord blood derived mast cells (CBMC). FcγRIIB displays different inhibitory effects on MC proliferation and activation. In murine BMMC, co-aggregation of FcγRIIB with FcεRI results in the inhibition of IgE-mediated mast cell degranulation. The intact ITIM in the cytoplasmic tail of FcγRIIB is crucial to this effect.[23,24] Similarly, co-aggregation of FcγRIIB with FcεRI inhibits degranulation and cytokine secretion. This response is mediated by phosphorylation of tyrosine residues on FcγRIIB and subsequent recruitment of SHIP-1 but nor SHP-1/2.[14,24-26] FcγRIIB can also negatively regulate mast cell proliferation when co-aggregated with Kit[27] through the activity of SHIP-1.

Siglecs

Members of the siglec family have one variable (V-type) region located at the N-terminal domain which binds sialic acid residues and between 1-16 C2-type Ig-like domains in the extracellular portion.[22] The majority of the members of this family contain ITIM or ITIM-like domains. These receptors are expressed on various cell types of the innate and adaptive immune systems. However, the siglecs that are expressed on mast cells belong mainly to the CD33-related siglecs.[28] Human mast cells express siglec-2, siglec-3, siglec-5, siglec-6, siglec-8 and siglec-10.[29] Notably, a relatively high level of siglec-5, 6 and 8 proteins, all of which contain one ITIM motif, have been detected on mast cells during allergic inflammation.[30-32]

Among the members of this family that are expressed on human mast cells, siglec-8 is the one that has been most studied. This receptor contains one ITIM and one ITIM-like domain that upon antibody-induced coligation of the receptor, undergo activation, recruit SHP-1 and trigger downstream inhibitory events. Although it was initially identified on eosinophils, where self aggregation by a mAb led to apopotosis, ligated siglec-8 on mast cells was also described to significantly inhibit FcεRI-triggered histamine and prostaglandin D_2 release. Interestingly, it had no effect on the release of other synthesized mediators such as cytokines.[33,34] As with many of the inhibitory receptors expressed on human cells, murine counterparts also exist in the siglec family. However, the murine functional homolog of the human siglec-8, siglec-F, is not expressed on murine mast cells but is expressed on murine eosinophils. Although the natural ligand for siglec-8 has long been unknown, recent studies have proposed mucins as the potential ligand, based on their ability to engage with siglecs and to subsequently induce apopotosis on monocytes.[35,36] Moreover, in a more recent paper, it has been shown that eosinophils can undergo apoptosis when incubated not only with anti-siglec-8 Abs, but also with a soluble synthetic polymer displaying 6′-sulfo-sLe^x glycan.[37]

CD300a

CD300a (IRp60 or CRMF-35) is expressed on NK cells, MCs, T-cell subsets, granulocytes, monocytes and dendritic cells.[15,38-40] It is one of seven members in a family of stimulatory as well as inhibitory Type I transmembrane glycoproteins that control and modulate leukocyte responses. CD300a contains four ITIMs in its cytoplasmic tail, three of them being "classical" (LHYANL, VEYSTV, LHYASV) and the fourth "non classical" (SDYSVI).[15] The function of CD300a was first described in NK cells where its co-aggregation resulted in down-regulation of the cells' cytolytic activity. Subsequently, CD300a's expression and function was further characterized in human mast cells. In CBMC, co-aggregation of CD300a with IgE-bound FcεRI, led to inhibition

of IgE-induced (but not of compound 48/80-induced) β-hexosaminidase, tryptase and IL-4 release. Concomitantly, an increase in CD300a phosphorylation, recruitment of SHP-1 and SHIP-1, decrease in Ca^{2+} influx and increase in syk dephosphorylation were detected. Moreover, CD300a cross-linking inhibited SCF-mediated CBMC survival.[38] Similarly, two bi-specific antibodies α-IgE/α-CD300a and α-Kit/α-CD300a, produced to crosslink the activating with the inhibitory receptors, abrogated Kit-mediated CBMC differentiation, survival and activation; and IgE dependent activation. In the context of mast cell-related disorders, when added to the malignant human mast cell line HMC-1, where Kit is constitutively activated, the CD300a bispecific antibody inhibited mediator release without affecting cells survival. In CBMCs, the inhibitory effect of CD300a on Kit was found to be mediated by a rapid phosphorylation of CD300a, recruitment of SHIP-1 but not of SHP-1 and by subsequent dephosphorylation of Syk and LAT.

CD300a and other members of this family have mouse orthologs, named CLM or CMRF-like molecules. The murine homologue of CD300a, LMIR-1 (or CLM-8), shares almost 80% homology with the human receptor and is expressed and functional on murine BMMC. In these cells, CD300a/Lmir-1 is capable of recruiting SHP-1, SHP-2 and SHIP.[41,42] CD300a has been shown to have a crucial role in the down-regulation or inhibition of allergic responses. This was demonstrated in murine models of allergic peritonitis, passive cutaneous anaphylaxis and acute asthma, where use of the murine bi-specific Ab α-CD300a(Lmir-1)/α-IgE proved to be effective in abrogating these allergic reactions.[43]

The ligand for CD300a remains unknown. However, evolutionary data reveals that CD300a is one of the human genes that shows strong positive selection[44,45] hinting that its potential ligand might have similarly gone through a strong positive selection as well.[46]

SIRP-α

SIRP-α belongs to the Ig-superfamily and is characterized by three Ig-like domains in the extracellular portion. The intracellular domain contains four tyrosine residues which form two ITIMs that recruit SHP-2 and SHP-1, thus negatively regulating signal transduction pathways.[47] SIRP-α has been shown to be expressed on human basophils and CBMC as well as on HMC-1 cells. A murine homologue of SIRP-α exists, but there is no evidence regarding its expression on mast cells. Functionally, SIRP-α inhibited mast cell degranulation when coligated with FcεRI and decreased the phosphorylation of FcεRI ITAMs. This was attributed to the recruitment of the tyrosine phosphatases SHP-1/2.[22] Moreover, it reduced intracellular Ca^{2+} mobilization, influx of extracellular Ca^{2+} and the activation of the MAP kinases Erk1 and Erk2. This resulted in inhibition of IgE-induced mast cell mediator release. The ligand SIRP-α has been identified as CD47, an integrin-associated transmembrane protein which is expressed on many cell populations. The interaction of the ligand with its receptor inhibits not only mast cell related responses but also FcγR dependent/independent phagocytosis by macrophages. Moreover, SIRP-α has been shown to inhibit production of IFN-γ by mature dendritic cells, suggesting that it inhibits the development of Th2 cytokines that drive allergic responses.

LILRs

LILRs belong to a family of receptors that are expressed on a wide range of cells such as B cells, dendritic cells, monocytes and NK cells. In humans, these receptors are

classified into three groups based on homology of Ig-like domains, gene architecture and organization: Ig-like transcripts (ILTs/CD85), killer-cell Ig-like receptors (KIRs) and leukocyte-associated Ig-like receptors (LAIRs (see below). It has been recently shown that LILRs are developmentally regulated in mast cells, suggesting a role in their maturation and differentiation. Human cord blood-derived progenitor mast cells (*hPrMCs*) express the inhibitory LILRB2, LILRB3 and LILRA2 on their surface. However, despite the presence of mRNA for multiple LILRs in mature CBMCs, LILR protein expression on the surface of these cells was not detected.[48] In contrast, flow cytometric analysis of mature human mast cells showed high intracellular expression of LILRB5 while none of the other inhibitory LILRs were detected.

Gp49B1 (mouse LILRB4), is a member of the Ig superfamily expressed constitutively on the surface of mast cells, neutrophils, macrophages[49] and natural killer (NK) cells. Gp49B1 is similar to FcγRIIB in that it contains two C2-type, Ig-like domains. However, gp49B1 has two cytoplasmic ITIMs.[50] The ITIM motifs in gp49B1 down-regulate NK cell and T-cell activation signals that lead to cytotoxic activity. In mouse BMMCs, gp49B1 binds SHP-1 and SHP-2 recruited from the cytoplasm upon tyrosine-phosphorylation. Gp49B1-mediated inhibition of FcεRI-driven activation is reduced in SHP-1-deficient BMMC.[51] Additionally, gp49B1 constitutively inhibits adaptive inflammation elicited by IgE-dependent mast cell activation in vivo.[52] It has also been shown that injection of LPS into gp49B1 null mice leads to hemorrhage, thrombosis and tissue neutrophilia. These results indicate that gp49B1 suppresses LPS-induced inflammation, thereby providing critical innate protection against a pathologic response to a bacterial component.[53] The ligand for gp49B1 has been identified as the integrin αvβ3, based on in vitro cell-cell and cell-protein binding studies. The interaction of αvβ3 with Gp49B1 on BMMC has been shown to inhibit antigen-induced IgE-mediated cell activation.[54]

Paired Ig-Like Receptor B (PIR-B)

PIR-B is an inhibitory receptor, originally identified in mice that belong to the family of the immunoglobulin-like receptors. PIR-B is expressed by many types of hemopoietic cells, including B lymphocytes, dendritic cells, monocyte/macrophages, granulocytes, megakaryocytes/platelets and mast cells.[55] This inhibitory receptor, as well as its activated counterpart, is produced by murine mast cells. PIR-B was found to be preferentially expressed on the cell surface, where it is constitutively tyrosine phosphorylated and associated with iSHP-1. After coligation with FcεRI, PIR-B inhibited IgE -mediated mast cell activation and release of serotonin.[56] The human counterparts of PIR-A and PIR-B are considered to be the activating and inhibitory types of leukocyte Ig-like receptors/ CD85. Out of four ITIMs that the PIR-B receptor contains, two of them were found to be able to recruit SHP-1 and possibly SHP-2 when tyrosine phosphorylated and this leads to inhibition of cell activation.[57] The ligand for PIR-B appears to be various mouse major histocompatibility complex class I (H-2) molecules. Indeed, stimulation of PIR-B with H-2 tetramer on B cells, leads to intracellular phosphotyrosine signaling.[58]

LAIR-1

LAIR-1 (CD305) is a human Type I transmembrane glycoprotein which contains a single extracellular C2-type Ig-like domain and two ITIMs in its cytoplasmic tail. Although most inhibitory receptors are usually restricted to specific cell types, LAIR-1

is expressed on almost all cells of the immune system. In the RBL-2H3 mast cell line, cross linking of LAIR-1 was able to inhibit FcεRI-mediated degranulation. Both ITIMs of this receptor are required for the full inhibition of degranulation in these cells, although the receptor is still partially active with one functional ITIM.[59] The murine homologue of LAIR-1 (mLAIR-1) shares 40% identity with the human receptor. Unlike many inhibitory receptors whose ligands have been identified, the ligand for LAIR-1 is not a cell-bound molecule. Collagens which are now known as the ligands for LAIR-1, define a new key role for extracellular matrix (ECM) proteins in immune regulation.[60] Human LAIR-1 recruits SHP-1 and SHP-2[61,62] but not SHIP.[63] However, mLAIR-1, recruits SHP-2 but not SHP-1. The inhibitory function of LAIR-1 is not completely phosphatase-dependent but can also be mediated through Csk, a negative effector that can inactivate Src family kinases, in cells where the phosphatases activity is abrogated or limited.[63]

CD200 Receptor (CD200R)

CD200R is a Type-I membrane glycoprotein, containing two Ig-like domains and which is expressed on MC[64] and basophils.[65] Unlike the majority of immune inhibitory receptors bearing ITIMs, CD200R does not contain a classical ITIM motif but instead contains three tyrosine residues in its cytoplasmic tail which may be critical for function. Previous studies have shown the expression of CD200R on human CBMC and skin mast cells. CD200R has also been detected on murine bone marrow derived mast cells (BMMC) and murine skin mast cells.

In human mast cells, CD200R produced an inhibition in degranulation that was enhanced by cross linking the anti-hCD200R Abs. This inhibition did not require coligation to an activating receptor such as FcεRI, although this did further enhance inhibition. This differs from most inhibitory receptors whose inhibitory effect depends on co-aggregation/ coligation to the balancing activating receptor. In murine mast cells, engagement of CD200R by its soluble ligand (CD200) did not lead to the inhibition of mast cell degranulation or cytokine production. However, overexpression of the receptor on these cells made them sensitive to anti-mCD200R Abs mediated inhibition. This demonstrates that CD200R activity depends on its density across the cells surface. Furthermore, the inhibitory effect of CD200R was also evident in vivo in a model of passive cutaneous anaphylaxis (PCA). Injection of anti-mCD200R Abs into mice (i.v) prior to mast cell activation significantly decreased PCA skin reactions in a dose dependent manner.

As mentioned, CD200R does not contain an ITIM sequence in its cytoplasmic domain. However, it has been shown that upon receptor engagement, CD200R is rapidly phosphorylated and recruits inhibitory adaptor proteins such as Dok1 and Dok2.[66] Two of the tyrosine residues of CD200R (Y286, Y297) have been found to be critical for the receptor- mediated inhibitory effect. One of these tyrosines is located in a conserved phosphotyrosine binding site ($NPXY^{297}$) for the signaling protein Shc. This motif mediates phosphorylation of Dok1 and Dok2 which subsequently bind RasGAP and SHIP, leading to a downstream inhibition of the RasMAPK pathways in the cell activation.

CD200 is strongly expressed on human and murine dermal fibroblasts, endothelial cells, dermal nerve bundles, hair follicles and subsets of glandular epithelial cells, but not on keratinocytes, Langerhans cells or the majority of mast cells and macrophages of the dermis in both murine and human skin sections.[67] Nevertheless, mast cells were

detected in close proximity to CD200-expressing cells in skin tissues of human skin and with a more significant expression in the murine skin.

(PECAM-1)

PECAM-1, also known as CD31, is a newly discovered member of the Ig superfamily containing six extracellular Ig domains and two ITIMs.[68,69] PECAM-1 was found to be expressed mostly at the lateral junctions of endothelial cells and at lower levels on neutrophils, monocytes, platelets, NK cells, T/B-cell subsets and on mast cells both of human and murine origin. This receptor has been shown to play a role in a number of biological processes, including leukocyte transmigration, cell migration, angiogenesis, cell adhesion, as well as modulation of intracellular signaling.[70]

In RBL-2H3 cells, following FcεRI clustering alone (without any need for receptor co-aggregation), PECAM-1 was shown to undergo rapid tyrosine phosphorylation on its ITIMs.[71] Based on plasmon resonance studies, it has been shown that PECAM's ITIMs bind SHP-2 with high affinity and SHP-1 with a lower affinity.[72] The ligands for PECAM-1 include the adhesion molecule $\alpha_v\beta_3$[73] (which is also the ligand for gp49B1 previously described) and CD38, a cell surface molecule involved in the regulation of lymphocyte adhesion to endothelial cells.[74]

The C-Type Lectin Superfamily

Mast Cell Function-Associated Antigen (MAFA)

MAFA is an ITIM-containing (1 ITIM) membrane glycoprotein which was first described in RBL-2H3 cells in which most of its inhibitory effects have been studied. Mouse and human homologues of the rat MAFA were thereafter discovered. Mouse MAFA was renamed as killer cell lectin-like receptor subfamily G member 1 (KLRG1), which exhibited an 89% similarity at the amino acid level to rat MAFA. As opposed to the rat receptor, KLRG1 was found to be expressed only by lymphokine- activated NK cells and by virus-activated CD8 T cells. It was not expressed on mice mast cells. Moreover, a human MAFA-like receptor has been identified as MAFA-L, sharing a 54% similarity to the rat receptor. The human receptor, differing from the rat MAFA, is expressed not only on mast cells and basophils, but also on various types of NK cells and the monocyte-like cell-line U937.

Human MAFA has been proposed to likely regulate responses to receptors other than the FcεRI.[75] In RBL-2H3 cells, MAFA has been shown to suppress the FcεRI secretory response and cytokine synthesis.[76] Aggregation of MAFA alone is sufficient to inhibit mast cell secretory responses prior to FcεRI activation. However, co-aggregation of MAFA with FcεRI has been recently shown to significantly increase the inhibition of mast cell degranulation.[77] Additionally, MAFA clustering has been found to negatively regulate the cell cycle of RBL-2H3 cells. This was evident by a significant increase in the numbers of cells arrested in the sub-G phase.[75] Using surface plasma resonance analysis it has been shown that the cytoplasmic tail of MAFA, containing an ITIM sequence, binds SHIP and SHP-2 but not SHP-1.[78] However, it has been shown that SHIP is the key phosphatase mediating the receptor's inhibitory effect. This involvement of SHIP is thought to mediate the recruitment of a multimolecular complex (Shc–SHIP–Dok–RasGAP) to the plasma membrane, where, RasGAP down-regulates the Ras-induced Raf-1/MEK/ERK signaling pathway by decreasing RasGTP levels. This in turn may eventually lead to a decrease in

gene transcription and in synthesis of cytokines regulated by Erk-1/2. On the other hand, according to various studies, MAFA does not interfere with the FcεRI-induced Fyn–Gab2–PI3K signaling pathway, which is essential for PKB and the Jnk pathway activation.

Regarding the ligand for MAFA, it was originally thought that the receptor binds MHC-I molecules. This hypothesis was suggested since there is a great homology between MAFA's extracellular domain and the CRD of other C-type lectins. However, there was no demonstrable interaction between murine MAFA and MHC-I or MHC-II molecules. It has also been implied that MAFA binds to saccharides following investigation of its binding capacity. Members of the classical cadherin family were shown to act as ligands for murine KLRG1[79-81] and the ligation of the receptor by E-cadherin led to an inhibition of CTLs induction as well as the lytic activity of an NK cell line in vitro. Recently, it has been demonstrated that the ligand for human MAFA is also the human E-cadherin.[82]

CD72

CD72, also termed Lyb-2, is an ITIM-containing, 45 kDa Type II transmembrane protein[83] predominantly expressed on B-lineage cells in both mouse and human.[84] The precise function of CD72 remains unclear. However, studies have shown that anti-mouse-CD72 mAb induces an increase in the metabolism of phosphatidylinositol in purified small splenic B cells.[85] Moreover, it induces an increase in MHC class II expression on B cells[86,87] and mobilization of small amounts of cytoplasmic free Ca^{2+} in those cells. The natural ligand for this receptor has been identified as CD100 or Semaphorin 4D (Sema4D).[88]

Recently, CD72 has been found to be expressed on human mast cell lines (LAD2, HMC1.1, HMC1.2) as well as on $CD34^{+}$ peripheral blood-derived mast cells. CD72 contains two ITIMs thus including this receptor in the inhibitory receptor family. Upon tyrosine phosphorylation, one of the receptor's ITIM binds SHP-1 and the other binds Grb2. On mast cells, coligation of CD72 with Kit by an anti-CD72 Ab (BU40) or the recombinant human CD100 (rCD100) and SCF respectively, led to CD72 phosphorylation and the subsequent recruitment of SHP-1. This resulted in dephosphorylation of SFKs and ERKs that are crucial in Kit-mediated human mast cell responses. CD72 ligation suppressed HMC1.2 growth and led to the reduction of Kit-dependent growth of human mast cells, SCF-induced chemotaxis, MCP-1(CCL2) production and SCF-enhancement of IgE-dependent degranulation. In contrast, CD72 ligation with FcεRI alone was unable to inhibit mast cell degranulation.[89]

INHIBITORY RECEPTORS AS THERAPEUTIC TOOLS?

Mast cell related diseases, such as allergic as well as non-allergic inflammatory diseases (asthma, rhinitis, conjunctivitis, etc.) affect a significant portion of the Western world's population with an increasing incidence each year. Some of these diseases might in certain cases lead to morbidity. Even with the proper use of current treatments such as glucocorticoids and several symptomatic drugs, symptoms and severe side effects may continue to occur and no ideal drug is yet available. Therefore, there remains a critical need for new approaches to treat mast cell-related diseases in a more useful and specific way. The expression of inhibitory receptors on mast cells makes them attractive targets for new drug designs for allergy and other mast cell-driven diseases. Indeed, some of the studies on inhibitory receptors aimed for therapeutic development are at an advanced stage.

With the discovery of the first inhibitory receptor, FcγRIIB, bi-specific antibodies recognizing both the receptor and FcεRI were generated. When tested in vitro, these antibodies showed a suppressive effect on basophil as well as on human mast cell activation.[90] Moreover, similarly to the bi-specific antibodies, the use of a fusion protein hFcγ-hFcε showed an inhibitory effect in both in vitro and in vivo studies using a murine passive cutaneous anaphylaxis model (PCA), as well as in skin test responses in Rhesus macaques allergic to dust mite.[91,92] For CD200R, in a murine model of PCA, treating the mice with specific antibodies targeting CD200R before challenge led to a significant dose-dependent decrease in cellular response.[67] Another member of the inhibitory receptors superfamily, CD300a has been an attractive target for the treatment of allergic diseases. Bi-specific antibodies targeting both mast cells and eosinophils have been generated and proven to be effective in abrogating allergic responses. Indeed, as described, in both in vitro and in vivo studies, the bi-specific antibodies α-IgE/α-CD300a and α-CCR3/α-CD300a abrogated allergic responses mediated by these cells.[39,42,43]

CONCLUSION

The field of inhibitory receptors continues to expand. This includes their application in the context of mast cells and allergic inflammatory diseases. Despite all the activity and research, many known inhibitory receptors are still not fully characterized on haematopoetic cells in general and on mast cells in particular. Therefore, there is a need for continued research to better characterize the existing inhibitory receptors, for example by studying their possible interactions with different activating receptors and discovering some of their unknown ligands. In addition, an effort should be made to identify other new inhibitory receptors on human mast cells.

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SECTION IV

MAST CELLS IN HEALTH AND DISEASE

CHAPTER 10

THE MAST CELL IN INNATE AND ADAPTIVE IMMUNITY

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Abstract: Mast cells (MCs) were once considered only as effector cells in pathogenic IgE- and IgG-mediated responses such as allergy. However, developments over the last 15 years have suggested that MCs have evolved in vertebrates as beneficial effector cells that are involved in the very first inflammatory responses generated during infection. This pro-inflammatory environment has been demonstrated to be important for initiating innate responses in many different models of infection and more recently, in the development of adaptive immunity as well. Interestingly this latter finding has led to the discovery that small MC-activating compounds can behave as adjuvants in vaccine formulations. Thus, our continued understanding of the MC in the context of infectious disease is likely to not only expand our scope of the MC in the normal processes of immunity, but provide new therapeutic targets to combat disease.

INTRODUCTION

The vertebrate immune system has evolved two main response systems designed to contain and eliminate microbial predation. The first, termed innate immunity, is a rapid, relatively nonspecific response that is designed to actively clear or contain pathogens.[1] The second response, called adaptive immunity, is a slower, precisely targeted response that develops over a period of a few days to weeks, resulting in life-long immunological memory against the invading pathogen.[2] The initiation of both of these responses during infection is dependent upon a rapid burst of pro-inflammatory mediators at the site of infection whose function is to activate and recruit various effector cells, such as neutrophils that

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dictate innate clearance of the pathogen and dendritic cells that initiate adaptive immune responses.[3] One cell type that may be integral to the development of this pro-inflammatory environment is the MC. MCs are granulated, tissue-resident leukocytes best known for their role in pathogenic inflammatory sequellae such as allergy.[4-7] However, numerous studies over the last 15 years have reshaped our perception of the MC to that of a beneficial first responder in the innate defense against bacterial infections.[8,9] Recently, this protective function has been extended to their role in the induction of adaptive immune responses during infection. This suggests that MCs have evolved to provide pro-inflammatory signals critical to the induction of both innate and adaptive immune responses during infection. In this chapter, we will evaluate the literature supporting three arguments that suggest vertebrate MCs are a beneficial first responder during infection in the absence of adaptive immunity. These arguments include their conserved localization in tissues throughout evolution, their diverse expression of pattern recognition receptors and their unique ability to rapidly create a pro-inflammatory environment in tissues. We will also evaluate the numerous studies demonstrating a role for the MC in the formation of both protective innate and adaptive immune responses.

THE STRATEGIC LOCALIZATION OF THE MAST CELL

One of the most attractive properties of MCs that support their role as a first responder to infection is their positioning not only within the tissue parenchyma, but their localization within the host organism. While MCs are present in most vascularized tissues, they are generally noted to be particularly abundant in tissues that are exposed to the environment.[10-13] This selective increase in MC density would presumably aid in the detection of microbes that use these sites as portals of entry into the host. While this concept has not been empirically evaluated, many studies have now demonstrated that mice containing reduced numbers of MCs or that are devoid of MCs are highly susceptible to infection.[8,9,14] Another means to assess the importance of MC placement in animals is to compare their location in different vertebrate species. In mammals, MCs are readily detected in the lung, intestines, skin and urogenital tract[10-13] and, in humans, have been described to reach densities of up to 500 to 4,000 per mm^3 in the lungs, 7,000 to 12,000 per mm^3 in skin and 20,000 per mm^3 in the gastrointestinal tract.[15] These densities have been noted to increase in the skin of humans that is not covered by clothing, suggesting that regions that experience continual environmental exposure respond by either recruiting more MCs or inducing their local proliferation.[16] Similarly, in lower order vertebrates, including amphibians, reptiles and birds, MC analogues that share structural and functional characteristics with their mammalian counterparts are found in the mucosa of the skin and intestines.[17-19] In different species of fish families, including salmonidis, cyprinids and erythrinids, MCs have been found in the skin, intestines and in the gills, a site that is continually exposed to pathogenic and environmental insult.[20-25]

Another common feature of MC placement in the tissues of different vertebrate species is that they are typically found deep beneath the epithelium in the mucosa, in close proximity to the blood vasculature. This selective placement of MCs near the vasculature may ensure that the release of MC derived pro-inflammatory products, including histamine and TNF, has near instantaneous effects on the local endothelium. In rodents, changes that MC-derived products may exert on local endothelium include increased leakiness between cells to promote edema and the induction of proteins that signal innate

effector cells, such as neutrophils or dendritic cells, to enter the tissue site to promote their antimicrobial functions. For instance, recent studies have found that MC release of tumor necrosis factor-α (TNF-α) at the local site of infection induces the expression of the adhesion molecule E-selectin on the local blood vessel endothelium.[26] The expression of E-selectin was required for the massive recruitment of dendritic cells into the infected tissue site and the subsequent induction of antibacterial humoral immunity.[26] Similarly, in fish, MCs have been noted in close proximity with capillary endothelial cells throughout the mucosa of the intestines, skin and the loose connective tissue of the gill arch.[21,27-30] As with rodents, injection of bacterial toxins into fish results in MC degranulation that is closely associated with the recruitment of neutrophils into the inflamed tissue site. Overall, the striking commonalities in different vertebrate species concerning strategic MC density and placement within tissues, suggests that common mechanisms have been retained throughout evolution to ensure that MCs are a first responder during infection.

THE MAST CELL MICROBIAL SENSORY ARRAY

In order for MCs to behave as first line responders against microbial predation, MCs must express a diverse sensory array of receptors that facilitate the recognition of pathogens, pathogen products or host components activated in the presence of pathogens such as complement. Moreover, these sensors must translate these signals into a pro-inflammatory context. MCs have long been known to use complement products to bind both Salmonella and the helminth *Schistosoma mansoni.*[31,32] MCs were also known to directly engage enterobacteria such as *E. coli* by binding FimH, the protein expressed at the tip of filamentous Type 1 fimbriae via a mannosylated receptor protein.[33] We now know that this protein is CD48 and engagement of *E. coli* through this receptor induces MCs to release pro-inflammatory cytokines such as TNF.[34] More recent studies have demonstrated that MCs can express a number of genetically encoded pattern recognition receptors (PAMPS) that recognize and bind conserved components of different pathogens such as lipopolysaccharide (LPS). These PAMPs include Toll-like receptors (TLRs) 1, 2, 3, 4, 5, 6, 7 and 9, whose expression appears to vary according to the MC model.[35-37] For instance, analysis of TLR expression profiles in fetal skin derived MCs and bone marrow derived MCs revealed that fetal-skin derived MCs express much higher levels of TLRs 3, 7 and 9 than bone marrow derived MCs. Moreover, several studies examining the TLR expression profile in human cultured MCs have revealed differential patterns of TLR expression that collectively include TLRs 1, 2, 3, 4, 5, 6, 7 and 9. Not surprisingly, engagement of TLRs with their ligands on cultured MCs induces the secretion of copious amounts of cytokines[35,36] that differ based on the TLRs that are activated. For instance, Kulka et al found that stimulation of human MCs with the TLR3 ligand poly I:C resulted in the production of IFN-α, but not TNF, IL-1β, IL-5 or GM-CSF.[35] By contrast, treatment of human MCs with several TLR2 activators induced substantial IL-1β and GM-CSF responses in the absence of a Type 1 IFN response.[38] Treatment of bone- marrow derived MCs with the TLR9 agonist CpGs resulted in the release of TNF, but not GM-CSF, IFN-γ, IL-4 or IL-12.[37] Recent studies have also found that stimulation of peritoneal derived MCs from mice with different TLR2 ligands can result in the production of differential cytokine responses. For instance, stimulation of peritoneal MCs with lipoteichoic acid (LTA) resulted in the production of IL-6, IL-1 and TNF-α, whereas treatment with macrophage activating lipopepetide-2 (MALP-2) resulted in the production of the anti-inflammatory cytokine

IL-10.[39] TLRs on MCs are also known to be functionally relevant to different primary challenges in vivo. For example, mice containing MCs deficient in TLR4 were found to be less able to survive in the cecal ligation and puncture (CLP) model of innate immunity than mice containing wild-type MCs.[40,41] This failure was linked to the inability of these mice to recruit neutrophils into the peritoneal cavity.[40,41] Mice containing MCs deficient in TLR3 were found to be unable to efficiently recruit T cells after injection of the TLR3 ligand polyI:C[42] and mice containing MCs deficient in TLR7 were unable to efficiently induce dendritic cell migration to lymph nodes after injection of the TLR7 agonist imiquimod.[43] Cultured human MCs have also been found to express dectin-1, a PAMP involved in the recognition of fungal pathogens. In this model, exposure of human MCs to yeast zymosan induced the production of the cysteinyl leukotrienes (LT)-C_4 and LTB_4, which was inhibited in the presence of a dectin-1 inhibitor.[44] Finally, MCs have recently been found to express the NLRP3 inflammasome, an innate PAMP expressed in the cytoplasm of cells.[45] MCs expressing an allele of NLRP3 responsible for cryopyrin-associated periodic syndrome (CAPS) were found to spontaneously release IL-1β that induced neutrophil recruitment and vascular leakage associated with the CAPS disease.[45] MCs are also activated by the adjuvant compound alum,[46] which has also been suggested to activate the NLRP3 inflammasome, however, this linkage has yet to be evaluated.

Studies have also evaluated the ability of MCs to engage humoral factors which comprise important components of the innate immune system. These include components of the complement cascade, LPS binding protein, C-reactive protein, pentraxins and collectins. Like PAMPs, such as TLRs, these circulating innate immunoproteins, which are evolutionarily conserved in both vertebrate and invertebrate species, are involved in both sensing of microbes and triggering of mechanisms to facilitate clearance of the infection. MCs in rodents and humans are now known to express several complement receptors including the C3aR,[47] C5aR,[48] CR2, CR4[49] as well as the α2β1 integrin which is a receptor for C1q.[50] In vitro, engagement of C3a was found to induce MC activation and chemokine production from human MCs.[51,52] In a murine model of septic peritonitis, C3-deficient mice were unable to induce MC-dependent neutrophil recruitment required for bacterial clearance.[53] Repletion of these mice with C3 restored MC-dependent effector functions.[53] Subsequent studies found that C3 likely engages the CR2 receptor on MCs, as mice containing MCs deficient in CR2 were unable to protect mice from septic peritonitis.[49] MCs are also able to bind several collectins, including mannose-binding lectin (MBL), surfactant protein A (Sp-A) and C1q complement protein through the α2β1 integrin. This interaction is required for MC- dependent responses against *Listeria monocytogenes* and the yeast cell wall component zymosan.[50]

MCs from multiple sources have also been shown to respond to the presence of peptides released from epithelial, endothelial or neural sources during infection or insult to the host. These include bradykinin,[54,55] substance P,[55,56] somatostatin,[55,57] adrenocorticotropic hormone (ACTH),[54,55] calcitonin gene-related peptide (CGRP),[55,58] corticotrophin-releasing hormone (CRH),[59] vasoactive intestinal peptide (VIP),[55,60] neurotensin,[61,62] neuromedin U[63] and peptide antibiotics such as β-defensins[64] and LL-37.[65-67] Many of these peptides evoke release of MC mediators including histamine,[59,64,66-68] cytokines and chemokines.[65-67,69,70] Functionally, neuromedin U has been implicated in mediating the MC-dependent inflammatory activities of complete freund's adjuvant in a footpad model of inflammation.[63] Responsiveness of MCs to these peptides is dependent upon their tissue localization, as MCs derived from the skin or serosal sources are generally more responsive to these peptides than those derived from the intestines.[55] Interestingly, MCs

do not always appear to express canonical receptors for each of these peptides. With the exception of the neurotensin and neuromedin U receptors, which have been identified on MCs, many of these peptide agonists of MCs have been suggested to stimulate MCs by crossing the cell membrane to directly activate pertussis toxin sensitive G ($G_i\alpha 2$ and $G_i\alpha 3$) proteins.[71] Alternatively, these peptides may act through Mas related gene (MRG)-X1 and X2 receptors on human cultured MCs or their rat analogues MRGB3 or MRGB8 on rat peritoneal MCs. MRG receptors are low affinity, nonspecific G-protein coupled receptors that can bind multiple cationic compounds and have been suggested to mediate the MC-activating properties of several host-derived peptides, including substance P, somatostatin and vasoactive intestinal peptide.[71] Overall, these many observations clearly indicate that MCs from rodent or human sources have the capacity to directly respond to the presence of diverse pathogens, their products, or indirectly through host components activated by the presence of pathogens or pathogen products. A comprehensive summary of bacteria, viruses, protozoan parasites, their products or host-derived compounds demonstrated to activate MCs in vitro, as well as known receptors expressed by MCs for some of these ligands are listed in Tables 1 and 2.

Table 1. Ligands that induce MC activation in vitro

Stimulus	Preformed	Cytokines/ Chemokines	References
Bacteria			
E. coli	+	+	148-151
K. pnuemoniae	+	+	148-149,151
K. oxytoca	+	-	149
S. aureus	+	+	149-150,152-153
S. epidermidis	+	?	149-150
S. cohnii	+	?	150
P. vulgaris	?	+	149
H. pylori	+/-	+	154-157
M. tuberculosis	+	+	158
H. influenza	+	?	152-153
S. pneumonia	+	-	82,159
S. viridians	+	-	152
S. agalactiae	+	?	150
B. catarrhalis	+	-	152-153
B. pertussis	?	+	160
S. typhimurium	+	?	31
B. burgdorferi	+	+	161-162
M. hominis	+	?	150
U. urealyticum	+	?	150
B. capillosus	+	?	150
A. naeslundii	+	?	150
L. fermentum	+	?	150
E. cloacae	+	?	149-150
P. aeriginosa	+/-	+	163-165

continued on next page

Table 1. Continued

Stimulus	Preformed	Cytokines/ Chemokines	References
Viruses			
Sendai virus	+	?	166
Dengue virus	-	+	167-168
Reovirus	-	+	169
HIV	-	+	170-171
Influenza Virus	+	?	172
Parasites			
S. mansoni	+	?	173
L. major	+	+	174
L. infantans	+	+	174
L. brasiliensis	+	+	175
T. spiralis TSL-1 antigen	+	+	176
Toxins			
Toxin A (*C. difficle*)	-	+	177
Cholera toxin (*Vibrio cholera*)	-	+	178-179
VacA (Cytotoxin from *H. Pylori*)	-	+	180
Hemolysin (*E. coli*)	+	+	181
Hemolysin (*S. marcescens*)	+	+	181-182
Hemolysin (*A. hydrophilia*)	+	+	181
Hemolysin (*L. monocytogenes*)	+	+	181
Pertussis Toxin (*B. pertussis*)	-	+	160
Listeriolysin O	+	+	183
Streptococcal pyrogenic exotoxin B	+	?	184
Streptolysin O	+	?	185
Staphylococcal enterotoxin B	+	?	186
TLR agonists			
PGN	+/-	+	40
PGN	-	+	38,90,92,187-188
Lipotechoic Acid	-	+	39
MALP-2	-	+	39
Lipophosphoglycan	-	+	189
Lipoarabinomannan	-	+	188
dsRNA, poly I:C	-	+	35,90,169
LPS	-	+	40,90,92,187
Flagellin	-	+	35
ssRNA, imiquimod, R848	-	+	90
CpG DNA	-	+	37,90,170
Zymosan	?	+	44
Host derived Products			
C3a	+	+	32,51-52,190-193
C5a	+	+	51,191,193-197
C1q	+	+	50,198
Collectins	+	+	50,199
Substance P	+	+	55,64,69-70,200-206

continued on next page

Table 1. Continued

Stimulus	Preformed	Cytokines/ Chemokines	References
Somatostatin	+	?	55,203-204,206
Vasoactive intestinal peptide	+	+	55,70,203-204,206
Neurotensin	+	?	55,61,200,205-209
β-defensins	+	+	64-66
LL-37	+	+	65-67
Endothelin-1	+	+	113,210-211
Bradykinin	+	-	55,205
Corticotropin releasing hormone	+	?	59,212

Pre-formed indicate MC mediators that are released from the MC granule such as histamine or β-hexosaminidase. Secondary mediators indicate mediators that are released after de-granulation, such as cytokines, chemokines or eicosanoids.
+ indicates a positive response. – indicates no response was detected. +/– indicates that both positive and negative responses have been reported. ? indicates that no such response was evaluated.

Table 2. The microbial sensory array of MCs

Receptor	Ligand	References
CD48	FimH	158,213
TLR1	Lipopeptide	38
TLR2	Peptidoglycan	38,40,90,92,187-188
	Lipotechoic acid	39
	MALP-2	39
	Lipophosphoglycan	189
	Lipoarabinomannan	188
TLR3	dsRNA/Poly I:C	35,90,169
TLR4	Lipopolysaccharide	40,92
TLR5	Flagellin	35
TLR6	Peptidoglycan, zymosan	38,40,92,187
TLR7	ssRNA/Imiquimod	43,90
TLR9	Bacterial DNA/CpGs	37,90,170
Dectin-1	Zymosan	44
C3aR	C3a	32,51-52,191-193
C5aR	C5a	194-196,214
CR2	C4b, C3b, iC3b, C3d	49,190
CR3	C4b, C3b, iC3b, C3d	14
α2β1 integrin	C1q, collectins, SP-A, zymosan	50
ETA	ET-1	113,210-211
NT receptor	Neurotensin	200
NMU-R1	Neuromedin U	63
MRG-receptors	Substance P, somatostatin, VIP	71

THE PRO-INFLAMMATORY ARSENAL OF THE MAST CELL

One of the most compelling arguments for the notion that MCs have evolved as first responders during infection is that they are uniquely equipped for the rapid release and synthesis of numerous pro-inflammatory mediators that have the capacity, either individually or in concert, to set in motion powerful inflammatory reactions. The types of pro-inflammatory compounds released by MCs have been divided into two major classes based on whether they are prestored or de-novo synthesized. Pre-stored compounds are packaged into cytoplasmic MC granules, which are the dominant feature of MCs in all vertebrates. These granules are enveloped by a membrane and are organized around a highly sulfated glycosylaminoglycan such as heparin or chondroitin sulphate.[72,73] Pre-stored components in rodent and human MC granules include soluble biogenic amines such as histamine and serotonin.[74,75] Numerous enzymes and proteases such as chymases, tryptases and carboxypeptidase A,[76-78] and cytokines such as TNF-α,[79] are also packaged within the granule and remain associated with the glycosylaminoglycan matrix after release of the granule into the tissue.[80,81] Other prestored compounds of note include antimicrobial peptides such as cathelcidin.[82] De novo synthesized mediators produced by rodent and human MCs include arachidonic acid-derived metabolites generated through the cyclooxygenase pathways such as prostaglandin (PG)-D_2 or the lipoxygenase pathways that yield leukotrienes such as LT-C_4.[76] MCs also secrete an amazing array of cytokines and chemokines, including IL-1,[40,83,84] IL-3,[83,85,86] IL-4,[40,83,85] IL-5,[87-89] IL-6,[40,83,87,90] IL-8,[89] IL-9,[87,91] IL-10,[92] IL-13,[40,89,92] GM-CSF,[89] TGF-β,[93] CCL1,[94] CCL2,[94] CCL3,[89,90] CCL4,[94] CCL5[90] and CXCL2.[90] Many of these compounds have been well documented to mediate the effects of tissue inflammation, including fluid extravasation and the recruitment of effector cells into the tissue.

In lower order vertebrates, the components of the MC granule and the secretion of de novo synthesized products are not as well characterized. However, it is generally thought that the granule components are similar. For instance, in different species of fish, the granules of MCs have been noted to contain serotonin,[95] lysozyme,[96] carboxypeptidase A5,[25] alkaline and acid phosphatases, arylsulphatase, 5-nucleotidase,[97,98] and potent antimicrobial peptides (called piscidins).[23] Notably, histamine has not generally been detected in the granules of fish or amphibian MCs, which is a characteristic thought to have started in the MCs of reptiles. However, recent studies have demonstrated that histamine is present in the MC granules of the Perciformes order, considered the most evolutionarily advanced order of fish. This suggests that the storage of this important pro-inflammatory compound in MC granules may have occurred as a separate evolutionary event. Functionally, activation of MCs in lower order vertebrates has been associated with inflammatory events in these species. Injection of inactivated bacteria into fish such as Atlantic salmon[99] or brown trout[28] elicit MC granule exocytosis followed by vasodilation and an inflammatory reaction.[100-102] Injection of fish with the MC secretagogue compound 48/80 elicits vasomotor responses in perfused brachial vascular beds of rainbow trout.[103] This last point is significant as mammalian MCs undergo rapid exocytic responses to compound 48/80, resulting in the induction of profound pro-inflammatory responses.[74,104] This point suggests that, not only do mammalian MCs and lower vertebrate MCs store common inflammatory components, but that they may also be hard-wired to respond to similar secretagogues.

The release of MC granules and their associated pro-inflammatory contents during microbial challenge is one of the principal ways in which MCs are thought to provoke the characteristics of inflammation. Exocytosis of the MC granule results in the instantaneous

release of many or the prestored pro-inflammatory products such as histamine. Interestingly, the granule itself is released intact into the local tissue, suggesting that its associated mediators such as TNF-α and many enzymes are retained within its matrix. This might ensure that the pro-inflammatory mediators remain undiluted and active so as to efficiently exert their effects within the tissue site. Recent studies in mice have confirmed this hypothesis, demonstrating that the MC granule conveys MC-derived TNF-α to distal sites such as the draining lymph node ensuring that its pro-inflammatory effects remain un-impaired.[105] Intriguingly, MC granule exocytosis has been demonstrated to be somewhat variable depending on the stimulus. These observations may reflect that the de-granulation event is not an all or nothing process, but rather an exquisitely tuned response that releases an appropriate amount of pro-inflammatory mediators appropriate for the stimulus. The first type of granule release is best defined after IgE receptor (FcεRI)-mediated crosslinking on the MC surface. In this model, MC granules begin to swell within minutes, followed by fusion of individual granules into cytoplasmic channels. The channels then fused with the plasma membrane resulting in an explosive expulsion of the granular material.[106,107] However, in the context of exposure to Type-I fimbriated *E. coli*, MC de-granulation occurs as a gradual exocytic event that takes an hour to complete.[108] Similarly, injection of *E. coli* into footpad tissues results in a gradual release of granule components from MCs over a four hour period, as opposed to the near immediate release of MC granules upon injection of the MC secretagogue compound 48/80.[109] The slow release of MC granules during infection is thought to reflect a form of granule release called piecemeal degranulation, which involves the slow release of granular material as the granule fuses with the cellular membrane followed by re-internalization of the MC granule. Interestingly, many bacterial products, including TLR ligands such as LPS or polyI:C, or toxins such as cholera toxin, do not induce MC degranulation.[35-37] Rather, as noted, exposure of MCs to these bacterial components results in the release of copious amounts of cytokines.[35-37]

MAST CELL MODULATION OF INNATE RESPONSES TO INFECTION

Overall, these observations suggest that MCs are well positioned and armed to be a primary point in the initiation of inflammation required for the induction of innate and adaptive immune responses during infection. Proof of this hypothesis, however, requires the use of in vivo models that facilitate the direct analysis of MCs and their impact on these responses during infection. Fortunately, certain mutant mice virtually lacking MCs are available, making it possible to evaluate the specific contribution of MCs. These MC-deficient mutant mice include the WBB6F1-Kit^{W}/Kit^{Wv} (W/Wv) mouse, a heterozygous mutant that bears a deleted *kit* allele and a mutant *kit* allele with defective kinase activity.[110] Recently, the Kit^{Wsh}/Kit^{Wsh} mice have been described as an alternative model to the W/Wv strains. These mice are preferable as they do not manifest some of the severe deficiencies of the W/Wv strain of mice, including profound anemia and sterility.[111] By quantitating differences in biological responses between MC-deficient mice and the congenic wild-type controls and then by analyzing the responses in MC-deficient mice that have been selectively reconstituted with cultured MCs, the specific in vivo contributions of MCs to many inflammatory reactions can be evaluated.[110]

Among the first studies that examined a role for MCs in the induction of innate immunity during infection in vivo were performed by Malaviya et al and Echtenacher et al in mice.[9,112] Using an infectious model of bacterial peritonitis, Malaviya et al found

that injection of FimH-expressing enterobacteria into the peritoneal cavity of W/Wv mice resulted in a near 80% mortality rate, as opposed to a 0% mortality rate in wild-type controls.[112] Repletion of the peritoneal cavity of W/Wv mice with cultured MCs restored the ability of these mice to resist enterobacteria induced mortality.[112] These data confirmed that the differences observed in susceptibility to infection were due to the absence of MCs and not to other abnormalities that may exist in these mice.[112] MCs were found to protect mice from enterobacterial challenge by recruiting neutrophils into the infected site, as MC-deficient mice exhibited a 5-fold reduction in the presence of neutrophils in the peritoneal cavity compared to MC-sufficient controls.[112] These findings were associated with the release of large amounts of local TNF-α, which was required for the recruitment of the neutrophils. Similar data, obtained using the cecal ligation and puncture (CLP) model of acute peritonitis, also found that MC-induced recruitment of neutrophils is important for protection against bacterial infection.[9] These data confirmed for the first time that MCs are an important component of innate defense based on their ability to recruit neutrophils into the site of infection.

Subsequent studies have expanded our understanding of how MCs affect innate defense in models not associated with neutrophil recruitment. The first is concerned with the limitation of the toxic effects of either host derived endogenous compounds, or exogenous mediators such as venom components that can have profound deleterious effects on host tissues. Original studies that examined this problem involved the endogenous peptide endothelin-1 (ET-1), which has significant adverse effects when present in high concentrations.[113] MCs and MC-derived carboxypeptidase A were found to be required to limit the morbidity and mortality induced by ET-1 administration in mice, as well as the effects of ET-1 in the CLP model of peritonitis.[113] Subsequently, MCs have been found to limit the toxic effects of the peptide neurotensin and the cytokine IL-6 through similar proteolytic activities.[62,114] Finally, comparative work found that the sequence of ET-1 was very similar to the toxic component of the Israeli mole viper sarafotoxin 6b. Because MC proteolytic activity was required to limit the harmful effects of ET-1, it suggested that MC proteolytic activity might limit the pathogenicity of sarafotoxin as well. Injection of sarafotoxin 6b into MC deficient mice demonstrated that these mice were 10 times more sensitive to the deadly effects of the venom than MC sufficient mice. Similar data was obtained using the venom from the pit viper (Crotalinae) and the honeybee (Apis mellifera),[115] suggesting that MC derived proteases can limit the toxicity of noxious substances.

An additional means by which MCs affect innate immune responses is through the release of prestored peptide antibiotics. Antimicrobial peptides are distinguished by their ability to rapidly inactivate a wide range of infectious agents.[116-120] These peptides are thought to act by disrupting the integrity of the microbial membrane,[117] a feature thought to be mediated by two common structural characteristics shared by most antimicrobial peptides, including a net-positive charge and the propensity for folding into amphipathic secondary structures that promote their binding and insertion into microbial membranes. The presence of antimicrobial peptides in MCs was first noted in hybrid striped bass.[23] These fish-associated antimicrobial peptides, now called piscidins, were found to be largely associated with MCs in the skin, gastrointestinal tract and the gills of nine different families of fish of the Perciformes order.[24,121] Piscidins comprise a family of 21-22 amino acid antimicrobial peptides that display a broad range of antimicrobial activity against many bacterial and protozoan pathogens.[24,121-122] These include not only disease-causing microbes in fish, but pathogens that cause disease in mammals such as *Candida albicans.*[123]

Subsequently, several antimicrobial peptides, including beta defensin-4, beta defensin AMP and cathelicidin were found to be expressed in both cultured human and murine MCs.[82] Similar to piscidins, these antimicrobial peptides display a wide range of antimicrobial activity. Interestingly, murine MC-derived cathelicidin was found to be important in protecting mice against infection with Group A streptococcus (GAS). For instance, injection of GAS into the skin of Wsh mice reconstituted with cathelicidin-deficient MCs exhibited GAS induced lesions of 120mm^2, compared to lesions of 25mm^2 in the skin of Wsh mice reconstituted with cathelicidin$^+$ MCs.[124] Therefore, the expression and release of antimicrobial peptides are likely an important weapon in the antimicrobial functions of the MC.

Collectively, these observations have been augmented by many in vivo studies that have evaluated the role of MCs, their microbial sensory array and/or MC pro-inflammatory products in models of innate host defense (Table 3). These studies have largely reaffirmed the idea that MCs and their products play a vital role in host defense against many different types of pathogens or toxins. There are however, several important exceptions to this general hypothesis. Infection of MC-repleted, MC-deficient mice with the enteric pathogen *S. typhimurium* in the peritoneal cavity did not improve the ability of these mice to resist infection. In fact, repletion of Wsh mice with MCs appeared to aggravate the severity of the disease and resulted in reduced mortality.[125] In addition, the few in vivo studies that have examined a role for MCs in protection from viral infection, including infection with Encephalomyocarditis Virus and Coxsackie B3 Virus, have suggested that MC deficiency results in reduced pathology of the disease.[126,127] However, reconstitution of Wsh mice with MCs restores the pathogenic effects of the viral illness.[126,127] In calves infected with RSV, MC activation and systemic release of their products appears to correlate with the severity of symptoms.[128-129] Finally, in models where large doses of TLR ligands are administered to mice, MC deficiency appears to ameliorate the pathogenic inflammation induced by these TLR ligands. For instance, MC deficiency reduced LPS-induced uveitis or cystitis and SM360320 (a TLR7 agonist) induced hypothermia.[130-132] These models offer a startling contrast to those that suggest MCs have a protective role. However, an explanation may lie in the fact that pathogens such as *S. typhimurium* and RSV are highly evolved to cause active disease in their respective hosts. This suggests an intriguing concept, that such pathogens may have evolved strategies to overwhelm or insidiously reprogram the MC to induce changes in the local tissue site that allow it to gain access and systemically infect the host. In addition, large, systemic amounts of TLR ligands reflect a state of overwhelming sepsis, which may result in unabated stimulation of MCs leading to persistent inflammation of the tissue site and/or systemic tissue edema and shock, similar to what is observed in IgE-mediated, MC-dependent systemic anaphylaxis. Therefore, depending on the type of microbial stimulus, MCs may exhibit beneficial or pathogenic responses.

MAST CELL MODULATION OF ACQUIRED IMMUNITY DURING INFECTION

Another compelling role for the MC has recently been suggested by the realization that the inflammatory context that drives the innate response is also pertinent to the induction of the adaptive immune response. Inflammation facilitates the enhanced recruitment of naïve antigen- specific T cells and antigen-laden antigen-presenting cells (APCs), most

Table 3. In vivo models examining a role for MCs and their products in host defense

Challenge	Site of Challenge	Host	MC Dependent Response	Refs.
Bacteria				
E. coli	Peritoneal cavity	Mice	Bacterial Clearance	8,215
E. coli	Bladder	Mice	Bacterial Clearance	216
K. pneumonia	Lungs	Mice	Bacterial Clearance	112
K. pneumonia	Peritoneal cavity	Mice	Bacterial Clearance	217
M. pulmonis	Lungs	Mice	Bacterial Clearance	218
C. rodentium	Intestines	Mice	Bacterial Clearance	219
S. pneumonia	Skin	Mice	Lesional size	124
H. pylori	Stomach	Mice	Bacterial Clearance	220
H. influenza	Middle ear	Mice	Bacterial Clearance	221
L. monocytogenes	Peritoneal cavity	Mice	Bacterial Clearance	222
F. novicida	Lungs	Mice	Bacterial Clearance	223
S. aureus	Peritoneal cavity	Mice	Bacterial Clearance	214
M. tuberculosis	Lungs	Mice	Bacterial Control	224
P. aeriginosa	Skin	Mice	Bacterial Clearance	225
CLP	Peritoneal cavity	Mice	Survival/Bacterial Clearance	226
CLP	Peritoneal cavity	Mice	Survival/Bacterial Clearance	40
CLP	Peritoneal cavity	Mice	Survival/Bacterial Clearance	49,227
TLR Ligands				
PGN	Ear	Mice	Lymph node hypertrophy	142
PGN	Skin	Mice	Vasodilation	40
Poly I:C	Peritoneal Cavity	Mice	T-cell recruitment	42
Imiquimod	Skin	Mice	Adjuvant activity	43
Viruses				
Newcastle Disease Virus	Peritoneal Cavity	Mice	T-cell recruitment	42
Parasites				
L. major	Skin	Mice	Parasite Clearance	228
L. major	Skin	Mice	No role	229
L. amazonensis	Skin	Mice	No role	230
T. spiralis	Intestines	Mice	Parasite Clearance	231
T. spiralis	Intestines	Mice	Parasite Clearance	232-233
N. brasilensis	Intestines	Mice	No role	234-235
P. burghei	Spleen	Mice	Parasite Clearance	236
S. ratti	Intestines	Rat	Parasite Clearance	237
H. longicornis	Skin	Mice	Parasite Clearance	238
Endogenous/ Exogenous Peptides				
Substance P	Skin	Mice	Inflammation	239
Endothelin-1	Peritoneal Cavity	Mice	Reduced toxicity	113
Neurotensin	Peritoneal Cavity	Mice	Reduced toxicity	62
Neuromedin U	Footpad	Mice	Inflammation	63
Sarafatoxin 6a	Peritoneal Cavity	Mice	Reduced toxicity	115

likely dendritic cells, into local draining lymph nodes. This massive recruitment of both cell types into lymph nodes ensures the maximal engagement of antigen-specific T-cell receptors on naive T cells with peptide/MHC Class II complexes on dendritic cells. In addition, inflammation induces the expression of costimulatory molecules (CD40, CD80, CD86) on dendritic cells, allowing them to efficiently stimulate antigen specific naive T cells.[3,133] Both of these signals provided by dendritic cells to antigen specific T cells are required for the efficient induction of antigen- specific immunity, the clearance of the pathogen and the formation of immunological memory. Therefore, the pro-inflammatory environment created by MCs during innate responses, may well have an important role in the induction of adaptive immunity.

Historically, most reports have not supported a role for MCs in the development of primary adaptive immunity.[5,134-138] These data are largely derived from contact sensitization models that did not find a role for MCs, largely in the challenge phase which automatically ruled out a role for the mast cell in the sensitization, or antigen presentation phase of the response.[139] Similarly, several studies employing prime boost regimens for the study of humoral response in MC-deficient mice found that these mice were capable of producing antibody responses, suggesting that MCs had no impact on the development of adaptive immunity.[5,134-138] However, a growing body of evidence has been convincingly derived from several lines of investigation employing in vivo mouse models implying that MCs do have an effect on the adaptive immune response. For example, MC-deficient mice were observed to exhibit impaired T-cell activation in a T-cell-dependent contact sensitization model.[140] The relevant experiment in this study demonstrated that transfer of splenocytes from MC-deficient mice primed with the contact sensitizer FITC failed to allow the development of a contact sensitization response after subsequent challenge of the ear with FITC compared to controls. These data suggested that, in the absence of MCs, inefficient sensitization priming of the FITC specific T-cell response occurred and that this was correlated with reduced dendritic cell migration from the epidermis. MCs were also found to be required for the full expression of the multiple sclerosis (MS) phenotype in the experimental autoimmune encephalitis (EAE) model of MS in mice. In these studies, the induction of EAE was found to be severely impaired in both the time-course and intensity in MC- deficient mice. It was subsequently found that T cells in this model exhibited a poorly activated phenotype after the administration of CFA and the MOG peptide.[141] In addition, several studies have demonstrated that MCs can elicit dendritic cell mobilization and accumulation in lymph nodes by crosslinking FcεRI or the injection of the TLR2 agonist PGN[138 142] or after mosquito bite.[143]

Initially, the search for a role for MCs in the development of adaptive immunity during infection began with linking MC activation to the enhanced sequestration of lymphocytes in draining lymph nodes. Lymph node swelling is a common occurrence in response to infection and is the result of the recruitment and entrapment of massive numbers of lymphocytes within a 24-48 hour time period.[3,133] This process is thought to increase the likelihood that rare antigen- specific lymphocytes will be appropriately stimulated by specialized antigen presenting cells (APCs) that have acquired fragments of the invading pathogen.[3,133] The first studies to examine this response used a modestly pathogenic strain of *E. coli* in a footpad model of infection in mice. Injection of *E. coli* rapidly elicited MC de-granulation, followed by lymph node swelling that peaked at 24 hours. This suggested that MC activation and release of their mediators, preceded lymph node swelling, raising the possibility that MC products could directly impact lymph node hypertrophy. To evaluate this, *E. coli* was injected into the footpads of MC

deficient mice; and it was found that *E. coli* failed to elicit comparable levels of lymph node swelling. Lymph node swelling could be repaired by reconstitution of MC-deficient footpads with cultured MCs. Importantly, it was found that MCs affected the sequestration of lymphocytes in the distal lymph node by the release of TNF-α that flowed into the lymph node and provoked the increased expression of adhesion molecules known to affect lymphocytes trapping such as V-CAM-1. This "remote control" of lymph node hypertrophy by MC-released TNF-α established that MCs could affect the entrapment of lymphocytes through communication with distal organs.[109] Subsequent to this study, several groups have found that the stimulation of MCs with different MC secretagogues can induce lymph node hypertrophy, although the MC mediators required are different.[138,142,143]

As noted, a second important arm of the primary immune response is the recruitment of antigen-laden DCs into lymph nodes during infection. During infection, DCs acquire pathogen- derived fragments in peripheral tissues and translocate to lymph nodes, where they find and activate rare antigen-specific lymphocytes.[144] Therefore, cues that affect DC homing to lymph nodes are very important to the development of primary immune responses. Interestingly, host- derived factors such as TNF-α are known to affect DC migration.[145] Because MCs produce copious amounts of TNF-α, we examined the idea that MCs might affect the migration of DCs to lymph nodes during infection. In these studies, injection of *E. coli* into the footpad of MC deficient mice resulted in markedly impaired DC accumulation in lymph nodes compared to wild-type controls. Reconstitution of the footpad site of MC-deficient mice with MCs repaired DC trafficking to lymph nodes. MC control over DC trafficking during infection was dependent upon MC-derived TNF-α, which was found to affect DC migration at two levels. The first was through the recruitment of DCs into the infected tissue site by increasing the expression of the adhesion marker CD62E on local blood vessel endothelium. The second was in the induction of DC chemo-attractants such as CCL21 in the lymph node. Therefore, MCs activated not only the local tissue site, but also the distal lymph node in order to increase the expression of proteins that were required for the recruitment of DCs into the tissue site followed by their egress to lymph nodes. A final point of this study was to establish that MCs were actually involved in the induction of primary immune responses during infection. Injection of *E. coli* into MC-deficient mice resulted in impaired antigen-specific immune responses compared to wild-type mice or MC-repleted, MC-deficient mice. Therefore, MCs are in fact required for the development of optimal antigen-specific immune responses through their ability to provoke the increased recruitment of DCs and lymphocytes into lymph nodes during an infection.[26]

The combination of these findings suggested that MCs might act at the pinnacle of the adaptive immune response by directing the mobilization of two of the important effector cells in the induction of adaptive immunity into draining lymph nodes. This led to the question, if one activated MCs in the context of a vaccine antigen, could you elicit an immune response? Subsequently, injection of the small molecule MC-activating molecule compound 48/80 (c48/80) with the vaccine antigen, protective antigen (PA) from *Bacillus anthracis,* in the footpad was found to generate rapid and profound anti-PA humoral responses. Injection of c48/80 plus PA failed to elicit robust responses in MC-deficient mice, implying that MCs were required for the adjuvant effects of c48/80. Not surprisingly, repletion of MC-deficient mice with MCs was able to repair the defect. An additional finding was that nasal instillation of MC activating compounds evoked antigen–specific secretory IgA along various mucosal surfaces, suggesting that MC activators could behave as potent mucosal adjuvants. This is significant because there are

very few safe adjuvants that can induce robust mucosal immunity along mucosal surfaces. These immune responses were not only robust, but functional, in that they provided neutralization in several lethal challenge models, including an in vitro anthrax lethal toxin assay and protected mice against a lethal vaccinia virus infection in vivo.[146] Subsequent studies found that c48/80 also acted as an effective adjuvant when administered though an intradermal route.[147] Therefore, MC-activating compounds are an effective and novel class of vaccine adjuvant.

CONCLUSION

Collectively, these numerous studies provide compelling evidence that MCs have evolved as a first line of defense to protect vertebrates against microbial predation. This hypothesis contrasts the notion that MCs are a vestibular remnant of evolution that is only capable of causing disease such as allergy. In support of these ideas are their consistent positioning in tissues of vertebrates, extensive microbial sensory array and ability to rapidly convert these signals into a pro-inflammatory context that in vivo studies have found to be important for innate and adaptive immunity during infection. However, it remains important to note that MCs may also play a negative role in the pathology of some infectious diseases and it will be important to determine how this occurs. Nevertheless, the clear beneficial context of the MC suggests that these cells might be manipulated for clinical benefit. As noted, MC-activating compounds have proven to be highly effective mucosal vaccine adjuvants and certainly, continued evolution of our understanding of the MC may result in tailored vaccine adjuvants that can provide the appropriate, protective immune stimulus for different pathogens. We can also envisage manipulating the MC to stimulate innate immune responses to therapeutically manage many different types of infection. Therefore, our continued study and understanding of this fascinating cell in the context of infection will likely provide insights leading to improved therapies for not only infectious disease, but many other pathological conditions where MCs have been suggested to play a role.

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CHAPTER 11

MAST CELLS AND IMMUNOREGULATION/ IMMUNOMODULATION

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Abstract: Mast cells often represent one of the first cells of the immune system to interact with environmental antigens, invading pathogens or environmentally-derived toxins. Mast cells also can undergo alterations in phenotype, anatomic distribution and numbers during innate or adaptive immune responses. In addition to their well-known roles as effector cells during IgE- and antigen-induced allergic reactions, mast cells can be activated by many other signals, including some that are derived directly from pathogens or which are generated during innate or adaptive immune responses. Mast cells also express many costimulatory molecules with immunoregulatory activities and can secrete many products that can positively or negatively regulate immune responses. In this chapter, we describe mouse models used for analyzing mast-cell function in vivo and illustrate how such models have been used to identify positive or negative immunomodulatory roles for mast cells during specific innate or adaptive immune responses. We also briefly describe some of the mast-cell functions, products and surface receptors that have the potential to permit mast cells to promote or suppress immune responses that can either enhance host defense or contribute to disease.

INTRODUCTION

Mature mast cells are long-lived tissue resident cells distributed widely throughout vascularized tissues. Large numbers of mast cells can be found near body surfaces in the skin, airways and gastrointestinal tract.[1-3] Mast cells, with dendritic cells (DCs) and monocytes, thus potentially represent one of the first cells of the immune system to interact

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with environmental antigens and allergens, invading pathogens or environmentally-derived toxins. Mast cells can re-enter the cell cycle and proliferate following appropriate stimulation and increased recruitment and/or retention and local maturation of mast-cell progenitors can also contribute to the expansion of mast-cell populations in tissues.[1-4] Expansion of mast cell numbers, local changes in their tissue distribution and alteration in their phenotypic characteristics can occur as a result of persistent inflammation and tissue remodeling.[1-4]

In addition to their well-known function in IgE-dependent responses, mast cells can respond to a variety of innate signals derived from pathogens, venomous animals, or the activation of complement, or from other host cell types, such as neurons.[5-9] Moreover, mast cells are equipped with a wide spectrum of costimulatory molecules that have immunoregulatory functions and also represent a potential source of many potent chemical mediators, growth factors, chemokines and cytokines, some of which can be rapidly released upon mast cell activation. With properties such as these, mast cells have the potential to regulate the transition from innate to acquired immune responses through effects that can either enhance or suppress the development, survival, proliferation, migration, maturation, or function of other immune cells.[2,3,6,7,10-13]

During individual biological responses, mast cells can function as effector cells, immunoregulatory cells, or both (Table 1). Mast cells can function as effector cells during innate[6,8,11,14,15] or acquired[6,7,10,12,13,16] immune responses. "Effector functions" of mast cells include killing pathogens,[11,14,15] degrading potentially toxic endogenous peptides[17-19] or components of venoms[18,20] and regulating the numbers, viability, distribution, phenotype or "non-immune" functions of structural cells, such as fibroblasts and vascular endothelial cells. Mast cells can exert effector functions through the direct or indirect actions of a wide spectrum of mast-cell-derived products and such effects can be observed in both innate[6,11,14] and acquired[6-8,10,12,13] immune responses.

Mast cells also can influence many aspects of the biology of immune cells, defined herein as cells of hematopoietic origin that participate in innate or acquired immune responses, including granulocytes, monocytes/macrophages, DCs, T cells, B cells, NK and NKT cells, etc. Effects of mast cells on the recruitment, survival, development, phenotype or function of immune cells are herein defined as "immunomodulatory functions".

Through effector and/or immunomodulatory functions, mast cells can promote the initiation and increase the magnitude of, the inflammation, tissue remodeling and, in some cases, tissue injury associated with immune responses, including innate or adaptive immune responses to pathogens as well as allergic or autoimmune disorders.[6-8,10-16,21] In vitro studies suggest that mast cells also can promote the development and extent of acquired immune responses through functions such as antigen presentation and many different effects on the biology of DCs, T cells and B cells.[12,15,22] However, few of these potential functions have been confirmed in vivo.

Given the many mechanisms by which mast cells can enhance the initiation or magnitude of immune responses, mast cells are often thought of as cells whose primary role is to "turn immune responses on". However, several lines of recent evidence indicate mast cells can also reduce the inflammation, tissue remodeling and tissue injury associated with immune responses.[12,13,23,24] Accordingly, a new picture of the mast cell is emerging: these cells have the potential to help turn immune responses off, as well as to turn them on. T cells specialized to down regulate immune responses are referred to as T regulatory (T_{Reg}) cells.[25] However, there so far is no evidence for the existence of a specific developmentally and phenotypically distinct "subset" of "immunoregulatory mast cells" specialized to down- regulate or suppress immune responses. Accordingly, we herein will refer to anti-inflammatory or

Table 1. Effector and immunomodulatory functions of mast cells[a]

Effector functions[b]
Promote clearance of pathogens by phagocytosis and/or secretion of antimicrobial peptides
Degrade potentially toxic endogenous peptides and components of venoms
Increase vascular permeability (e.g., by histamine)
Stimulate bronchial smooth muscle–cell contraction (e.g., by leukotriene C_4)
Promote fibroblast collagen synthesis (e.g., by tryptase)
Immunomodulatory functions[c]
Examples of positive immunomodulatory functions
Promote the migration, maturation, differentiation and function of immune cells via secretion of factors such as TNF, chemokines, histamine, LTB_4 and proteases
Present antigen to T cells (via MHC class I or II molecules) or enhance Antigen presentation by capturing IgE-bound-antigen via FcεRI and then undergoing apoptosis
Promote B-cell IgE production (through IL-4, IL-13 and CD40L)
Promote B-cell IgA production (through IL-6 and mast cell-B cell proximity)
Promote expression of TSLP on epithelial cells (for example, by TNF, IL-4 and IL-13)
Promote recruitment of immune cells by production of TNF and other mediators that upregulate adhesion molecule expression on vascular endothelial cells
Promote T_H2 responses via effects of prostaglandin D_2 on DC maturation
Promote airway smooth muscle production of chemokines and cytokines (via TNF, IL-4 and IL-13)
In the presence of physiological levels of IgE, promote sensitization in certain models of CHS (via increased migration of skin DCs and perhaps other functions)
In response to certain activators of mast cells used in conjunction with vaccines, enhance development of protective adaptive immune responses to pathogens
Examples of negative immunomodulatory functions
Suppress sensitization for CHS (via UVB-induced production of histamine)
Promote peripheral tolerance to skin allografts (via mechanisms that remain to be defined)
Mediate Anopheles mosquito-bite-induced suppression of development of certain T-cell-dependent responses (via effects of mast cells that remain to be defined)
Suppress many features of certain models of severe CHS, in part via effects of IgG1/mast cell-FcγR-dependent production of IL-10
Mediate jet fuel-induced suppression of CHS (via PGE_2 and perhaps IL-10)
Suppress many features of the pathology of chronic low-dose UVB irradiation of the skin, in part via effects of 1α,25-dihydoxyvitamin D_3-mast cell-VDR-dependent production of IL-10

continued on next page

Table 1. Continued

Suppress cytokine production by T cells and monocytes (via IL-10)
Suppress production of pro-inflammatory cytokines and chemokines by keratinocytes (via IL-10)
Enhance ability of DCs to reduce T-cell proliferation and cytokine production (via IL-10)

a) This table is modified from Galli SJ et al. Nat Rev Immunol 2008; 8:478-86 (ref. 13) with permission from Macmillan Publishers Ltd. Please see the original source for relevant references.
b) Effector functions include the non-immunomodulatory physiological or pathological functions of mast cells or the direct regulation of ''nonimmune'' cells, such as vascular endothelial cells, epithelial cells, fibroblasts, nerve cells and muscle cells.
c) These are effects on other immune cells (such as DCs, T cells, B cells, monocytes/macrophages and granulocytes) and effects on structural cells (such as vascular endothelial cells, epithelial cells and smooth muscle cells) that alter their ability to influence immune cells.

immunosuppressive functions of mast cells as "negative immunomodulatory" functions and to those functions that enhance the initiation, magnitude or duration of immune responses as "positive immunomodulatory" functions" (Table 1).

In this chapter, we highlight mouse models used for analyzing mast-cell function in vivo and illustrate how such models have been utilized to identify immunomodulatory roles for mast cells during specific immune responses. We also briefly describe some of the mast-cell functions, products and surface receptors that have the potential to contribute to the mast cell's ability to promote or suppress immune responses (reviewed in refs. 6,7,9-11,13,16).

MOUSE MODELS OF MAST-CELL FUNCTION

Mast-Cell Knock-In Mice

Although mice that specifically lack only mast cells have not been reported, c-*kit* mutant mice, which are deficient in mast cells but have several other phenotypic abnormalities, are available for analyzing the in vivo functions of mast cells.[1,6,26] The most commonly used animals for such studies are the WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice and C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice.[6,7,26-28] *Kit*^{W} is a point mutation that produces a truncated Kit, lacking the transmembrane domain, that is not expressed on the cell surface; *Kit*$^{W\text{-}v}$ is a (Thr^{660}→Met) mutation at the c-*kit* tyrosine kinase domain that substantially reduces the kinase activity of the receptor; and *Kit*$^{W\text{-}sh}$ is an inversion mutation that affects the transcriptional regulatory elements upstream of the c-*kit* transcription start site on mouse chromosome 5 (reviewed in refs. 6,7,28).

Adult WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice and C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice are profoundly deficient in mast cells and melanocytes.[1,6,26,27] WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice exhibit several other phenotypic abnormalities, such as macrocytic anaemia, reductions in numbers of bone-marrow and blood neutrophils, sterility and an almost complete loss of interstitial cells of Cajal.[1,6,26] By contrast, C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice are neither anaemic nor sterile, but have increased numbers of bone-marrow and blood neutrophils, enlarged spleens and mild cardiomegaly.[26,28-30]

Because the c-*kit*-related phenotypic abnormalities that affect lineages other than mast cells are generally milder in C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice than in $WBB6F_1$-$Kit^{W/W\text{-}v}$ mice and because C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice are fertile and, like many other mutant mice to which they might be bred, are fully on the C57BL/6 background, they are becoming increasingly popular for studies to elucidate the roles of mast cells in vivo. Herein, we will refer to both $WBB6F_1$-$Kit^{W/W\text{-}v}$ and C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice as "c-*kit* mutant mice".

Differences in the biological responses in c-*kit* mutant mice compared with wild-type mice may be due to any one of the genetic abnormalities in these animals and may not be due to the loss of mast cells. However, the lack of mast cells in c-*kit* mutant mice can be selectively repaired by the adoptive transfer of genetically-compatible, in-vitro-derived wild-type or mutant mast cells.[1,6,26,27] Such in vitro-derived mast cells, for example bone-marrow-derived cultured mast cells (BMCMCs), can be administrated to c-*kit* mutant mice intravenously, intraperitoneally or intradermally, or directly injected into the anterior wall of the stomach, to create so-called "mast-cell knock-in mice". These mast-cell knock-in mice can then be used to assess the extent to which differences from wild-type mice in the expression of biological responses observed in c-*kit* mutant mast-cell-deficient mice reflect their lack of mast cells.

Transgenic Mice with Deletion/Mutation of Mast Cell-Specific Products

The role of specific mast-cell-associated mediators can be investigated in vivo by testing animals in which that mediator has been knocked out. To the extent that the mediator is selectively expressed by mast cells and if its deletion does not significantly influence the expression of other mast cell products, then one can draw conclusions about the role of that mast cell mediator in vivo. For example, in mice that lack mouse mast-cell carboxypeptidase A (mMC-CPA, also known as mast cell-CPA and CPA3; a highly conserved secretory granule protease), the expression of mouse mast-cell protease 5 (mMCP-5) is also reduced because it requires mMC-CPA for proper packaging in the cytoplasmic granules.[31] This problem can be circumvented by using mice in which mMC-CPA has been mutated specifically to eliminate its catalytic activity, a change that preserves mMC-CPA's ability to ensure proper packaging of mMCP-5 in the mast cell granule.[20] Mice that lack mast cell protease-1 (MCPT-1),[32] MCPT-4,[33] tryptase beta 2 (TPSB2; also known as MCPT-6),[34] or mast cell-CPA/CPA3,[31] or that have a mutated form of mast cell-CPA/CPA3 that essentially lacks enzymatic activity,[20] have been used to analyze whether the absence of these proteases (or their enzymatic activity) influences other aspects of mast-cell phenotype, such as content of other stored mediators, and to define the functions of such proteases in vivo.

Transgenic mice expressing Cre-recombinase under the control of "mast cell specific" promoters recently have been generated.[35-37] Such "mast cell cre mice" are being crossed with other transgenic mice in which the genes of interest are "floxed" in attempts to reduce the expression of specific gene products only (or, at least, predominantly) in the mast cell lineage. Such approaches may prove to be useful in attempts to analyze to what extent mast cells represent important sources of products (including those with potential effector and/or immunomodulatory functions) that can also be derived from other cell types. "Mast cell cre" mice could also be mated to other transgenic mice in which important mast cell survival factors are floxed in order to ablate mast cells selectively. This approach may permit the generation of "improved" mast cell-deficient mouse models that are independent of c-*kit* mutations. However, time will tell whether various "mast cell cre" mice achieve

truly mast-cell-specific expression of Cre recombinase activity, or can be used to ablate all mast cell populations without affecting other cell lineages.

Other Approaches

Pharmacological approaches or those based on the use of antibodies to deplete mast cells or to neutralize their products may also provide useful information, but are limited by the specificity of the drug or antibodies chosen. Some agents, such as antihistamines, block the effects of that mediator whether it is secreted from mast cells or other cells. Antibodies that neutralize SCF[38] or block Kit[39-41] can result in the depletion of mast cells in vivo, but may also influence other cell types that express Kit.

Drugs (or antibodies) that only interfere with mast-cell activation would be highly desirable for experimental studies and, possibly, for evaluation as therapeutic agents. One drug, disodium cromoglycate, is widely characterized as a "mast-cell stabilizer" (i.e., an agent that blocks the release of mast cell mediators that occurs upon appropriate activation of the cell) and sometimes is used to suppress mouse mast-cell function in vivo,[42,43] but its molecular targets are not fully defined. However, these targets are not restricted to mast cells[44] and the drug also influences granulocyte and B-cell function.[45] Given the current limitations of using pharmacological or antibody-based approaches to eliminate mast cells or specifically to block their functional activation, we think that genetic approaches, including those employing mast cell knock-in mice, mice deficient in specific mast-cell-associated mediators and, when they have been fully validated, approaches that genetically delete specific mediators selectively in mast cells, are the most definitive way to identify and characterize mast cell functions in vivo.

MAST CELL ACTIVATION

Mast cells can be activated by a wide range of stimuli, including those that activate immunoglobulin Fc receptors (FcεRI, FcγR), complement receptors (C3aR, C5aR) and microbial pattern-recognition receptors (PRRs, such as TLRs). Mast cells also respond to many other signals, including neuropeptides, cytokines, growth factors, toxins, venoms or venom components and physical stimuli. These stimuli trigger mast cells to release a diverse array of biologically active products, many of which can potentially mediate pro-inflammatory, anti-inflammatory and/or immunosuppressive functions.[13] The strength and nature of the responsiveness of mast cells to various activating stimuli may be influenced by intrinsic or microenvironmental factors that affect the expression pattern or functional properties of the surface receptors or signaling molecules that contribute to such responses.[6,46-48] Furthermore, mast cells can participate in multiple cycles of activation for mediator release and can be differentially activated to release distinct patterns of mediators or cytokines, depending on the type and strength of the activating stimuli.[6,49]

Activation via FcεRI and Other Fc Receptors

Mast cells and basophils are the two major cell populations in the mouse that constitutively express on their surface large numbers of the high affinity receptor for IgE, FcεRI and the number of surface FcεRI is up-regulated by increased concentrations of IgE.[3] Aggregation of FcεRI by binding of bi- or multi-valent antigen to surface- FcεRI

-bound IgE on mast cells initiates complex intracellular biochemical events that lead to degranulation and secretion of mast-cell-derived products and mediators. This FcεRI-dependent mast cell activation response results in rapid release (in minutes) of preformed cytoplasmic granule-associated mediators (such as histamine, heparin and other proteoglycans, proteases) and certain cytokines (TNF, VEGF), the secretion of de novo-synthesized lipid mediators (including cysteinyl leukotrienes [LTs] and prostaglandins [PGs]) and the production, with a prolonged kinetics, of many cytokines, chemokines and growth factors.[3,6,50]

Aggregation of only a small fraction of the mast cell's FcεRI is sufficient to trigger mast cell activation and mediator secretion; as a result, individual mast cells can be simultaneously sensitized to respond to many different specific Antigens.[3] As discussed above, the extent to which mast cells secrete various types of mediators can vary according to the strength of the activation signal, with release of some cytokines occurring at lower antigen concentrations than the concentration required to induce substantial degranulation and release of stored mediators.[49] For example, low occupancy or weak stimulation of FcεRI can induce mast cells to produce "pro-allergic" chemokines, while mast cell IL-10 production requires strong and prolonged stimulation of FcεRI.[51] The extent of mast cell activation in response to FcεRI stimulation can also be positively or negatively regulated by the cells' exposure to ligands for many other receptors.[6,49,50]

Antigen- and IgE-dependent mast cell activation is widely regarded to be a, if not *the*, major initiator of the clinical signs and symptoms of an allergic reaction and can also contribute to later consequences of allergen exposure, by promoting local inflammation and by directly or indirectly enhancing certain aspects of tissue remodeling. In addition to FcεRI, mast cell activation can also be triggered by Fcγ receptors. IL-4 primed-mouse mast cells can produce TNF, IL-10 and VEGF upon IgG1/FcγRIII dependent activation[24,52,53] and aggregation of FcγRI on IFNγ-treated human mast cells can induce histamine release.[54]

TLRs and Other Innate Receptor-Mediated Activation

Mast cells activated by innate signals also can influence the development of acquired immune responses.[7,13,55-62] Mast cells express many Pattern Recognition Receptors (PRRs).[63-65] Protein or mRNA of 10 TLRs (TLRs 1–10) have been detected in human or mouse mast cells.[65] Mast cell responses to TLR agonists can vary among different populations of mast cells. For example, the stimulation of TLR4 on mouse mast cells by LPS resulted in cytokine production without degranulation, while TLR2 activation led to both cytokine release and degranulation.[66] Mast cells activated by TLR3 stimulation (e.g., with Poly(I:C) or Newcastle disease virus) produced antiviral cytokines (IFN-β, ISG15) and chemokines (IP10, RANTES) without undergoing degranulation.[67] Human and mouse Mast cells express NLRs (the second major class of cytoplasmic innate immune sensors), such as NOD receptors and NLRP proteins,[68-71] which can be activated by intracellular pathogens and "danger signals".[72-74] Expression of various PRRs thus permits mast cells to respond to both Pathogen-Associated Molecular Patterns (PAMPs) and "danger signals" resulting from cell stress or injury. Moreover, studies in mice indicate that activation of mast cells via the NLRP3 inflammasome can contribute to IL-1β overproduction and chronic urticarial rash in subjects with cryopyrin-associated periodic syndrome, a disorder associated with NLRP3 mutations.[71]

In conclusion, mast cell secretion of cytokines and other mediators in response to nonFcR-dependent innate stimuli, such as to TLR agonists, complement products and certain inflammasome activators, has the potential to influence the phenotype, maturation and function of Antigen Presenting Cells (APCs) and other immune cells and thus to modulate the subsequent T-cell or B-cell responses.[6-8,12]

IMMUNOMODULATORY EFFECTS ON DENDRITIC CELLS

Both in vitro and in vivo evidence indicate that mast cells have the potential to influence DC migration, maturation and function.[6,7,13] Histamine, abundantly stored in mast cell granules and rapidly released by the process of mast cell degranulation, can induce chemotaxis,[75] alter the pattern of secreted cytokines[76-78] and enhance expression of costimulatory and MHC class II molecules in DCs;[77] and histamine-conditioned DCs have been shown to preferentially induce T_H2 polarization.[76,77] In vitro coculture of IgE+antigen-activated umbilical cord blood-derived human mast cells with human monocyte-derived DCs induced maturation of DCs, but potently suppressed IL-12p70 production by DCs and thus promoted their T_H2 polarization.[79] Other mast cell products, such as PGD_2[80] or PGE_2,[81] have also been shown to inhibit IL-12 production by DCs and to induce maturation of DCs toward an effector DC2 phenotype, again leading to the polarization of naive T cells to T_H2 cells. These in vitro observations are in accord with in vivo findings showing that antigen immunization in conjunction with the administration of agents that induce mast cell degranulation suppresses antigen specific T_H1 responses while enhancing T_H2 responses.[82] Many other mast cell products, such as PGD_2,[80,83,84] LTB_4;[85] and PGE_2, TNF, IL-1, IL-16, IL-18 and CCL5 (reviewed in ref. 7), have also been shown to modulate DC migration, differentiation, maturation and function.

Another mechanism by which mast cells can regulate DC function is via exosomes, small vesicles implicated in the transfer of materials among cells. Mecheri and his colleagues have shown that endocytosed antigens are accumulated in association with heat shock protein hsp60 and hsp70 in mast cell exosomes. Such mast cell-derived exosomes are highly potent in inducing maturation (CD40 and CD80 expression) and functional activation (secretion of IL-12p70) of DCs and can potentiate the efficiency of antigen presentation by DCs.[86]

Relatively few studies have investigated the ability of mast cells to influence DC biology in vivo. In a model of contact hypersensitivity (CHS) to oxazolone, Bryce et al reported evidence that the emigration of skin Langerhans Cells (LCs) from the epidermis in response to epicutaneous application of oxazolone was impaired in mast-cell-deficient $WBB6F_1$-$Kit^{W/W\text{-}v}$ mice and was enhanced by antigen-independent effects of IgE.[87] Moreover, this group showed that mice lacking IgE exhibited impaired elevation of mRNA for the mast-cell-associated product, MMCP-6 (also known as MCPT-6 or TPSB2), as well as for several products (that can be produced by mast cells and other cell types) known to influence DC biology (such as IL-1, IL-6, CCL2 and TNF), one hour after the epicutaneous application of the hapten oxazolone.[87] These results suggest that the binding of IgE to FcεRI on dermal mast cells, even in the absence of antigen known to be recognized by that IgE, in some way can "prime" such mast cells to participate more effectively in the induction of DC migration and perhaps other functions that promote sensitization to the hapten.[87] Histamine derived from IgE- and antigen-stimulated mast cells in mouse skin can promote the H2 receptor-dependent migration of LCs to draining lymph nodes (LNs)[88]

and TNF derived from mouse mast-cells can contribute significantly to the initial stages of FITC-induced migration of cutaneous and airway DCs.[89] Administration of peptidoglycan can result in mouse mast cell-dependent LN hypertrophy, LC mobilization[90] and recruitment of plasmacytoid and $CD8^+$ DCs to draining LNs.[91] While peptidoglycan-induced LN hypertrophy was TNF-independent in this model, optimal LC migration required TNF.[90] Activation of dermal mast cells by a TLR7 agonist also has been shown to promote the emigration of LCs, via a process partially dependent on mast-cell-derived IL-1.[56]

IMMUNOMODULATORY EFFECTS ON LYMPHOCYTES

Evidence for the migration of mast cells to draining LNs during immune responses and the close proximity of mast cells and lymphocytes at sites of tissue inflammation[6,12,92,93] suggest that mast cells and lymphocytes may influence each other's functions by bidirectional cell–cell interactions.

Regulation of Mast Cell Functions by T Cells

Upregulation of IL-8 mRNA transcription has been demonstrated in mast cells that were in contact with the membrane of activated T cells.[94] Incubation of mast cells with activated lymphocytes induced mast cell degranulation and release of granule-associated mediators/proteases (histamine, β-hexosaminidase, metalloproteinase-9 and tissue inhibitor of metalloproteinase [TIMP]1), TNF and other cytokines.[95-98] ICAM-1/LFA-1 dependent cell-cell contact is necessary for anti-CD3-activated T cells to augment FcεRI-dependent mast cell (BMCMC) degranulation and histamine release[96] and the interactions between $LT\alpha1\beta2$ and/or LIGHT on activated T cells and LTβ receptors expressed on mast cells are important for mast cell cytokine release induced on contact with activated T cells.[98]

Mast Cell Effects on T-Cell Functions

Mast cells can also modulate T-cell functions. Antigen processing and presentation has been proposed as one mechanism by which mast cells might regulate T-cell responses.[7,92,99] Mecheri and his colleagues demonstrated upregulation of MHC class II in BMCMCs upon stimulation with LPS and reported that BMCMCs can take up, process and present antigen peptide to antigen specific T hybridoma cells.[22] Rat peritoneal mast cells (PMCs)[100] and human mast cells[101] were also later reported to be capable of antigen presentation. MHC class I molecules are expressed on mouse BMCMCs and on human mast cells isolated from lung, liver, uterus and skin[99] and, in vitro, mouse BMCMCs can stimulate bacterial antigen–specific CD8 T-cell activation by presenting bacterial antigens through MHC class I.[102] Recently, Stelekati et al showed that BMCMCs can present antigen to CD8 T cells in a MHC I-restricted manner, resulting in IL-2, IFNγ and MIP-1α production and promoting CD8 T-cell degranulation and cytotoxicity.[103] Although less efficiently than professional APCs, MHC II-expressing mast cells can upregulate CD69 expression, proliferation and cytokine production in effector T cells.[104,105]

The expression of MHC molecules and antigen presenting functions in mast cells appear to be under complex control: MHC II expression is down-regulated by IL-3, but upregulated by IL-4 and IFN-γ.[101,104,106] GM-CSF, while not influencing expression of MHC II, can increase CD80 and CD86[107] expression and substantially enhance the

antigen-presenting capacity of IL-4-treated mast cells. By contrast, IFNγ almost completely abolished mast cell antigen-presenting function.[106] In another study, Delta-like 1 (Dll1)/Notch signaling was shown to induce the expression of MHC-II and upregulate the expression of OX40L on mast cells, thus promoting the ability of MHCII^{+}OX40L^{high} BMCMCs to enhance naïve T-cell proliferation and their differentiation into IL-4, IL-5, IL-10 and IL-13 secreting T_H2 cells.[108] Treatment of cultured mast cells (BMCMCs and spleen-derived mast cells) with LPS/IFNγ also enhanced mast cell expression of MHC-II and the inhibitory costimulatory molecule PD-L1, but not positive costimulatory B7 family members CD80 and CD86 and promoted the mast cells' ability to process and present antigen directly to previously activated effector CD4 T cells and, to a lesser degree, to naïve T cells. Furthermore, LPS/IFNγ-primed mast cells stimulated expansion of antigen-specific Foxp3^{+} T_{Reg} cells preferentially over naïve T cells.[105] This finding may have implications for understanding the mechanisms by which mast cells can exert anti-inflammatory functions in certain T-cell mediated acquired responses.[24,109]

Although MHC class II molecules are not expressed on most "resting" mouse or human mast cells, expression is upregulated in mast cells that have been isolated from pathogen-infected tissues and/or stimulated by tumor necrosis factor (TNF), IFNγ or bacterial lipopolysaccharide (LPS).[99,105] Kambayashi et al showed that s.c. injections of LPS increase mast cell numbers in the draining LN and that these mast cells expressed MHC II, PD-L1, CD80 and CD86.[105] Freshly isolated peritoneal mast cells and IFNγ/IL-4-primed mast cells, derived from peritoneal cell culture, have been shown to process protein antigen and to form functional immunological synapse with and induce activation of, effector CD4 helper T cells, but not naïve T cells.[104] The cognate interactions at the immunological synapse between T helper cells and antigen-presenting mast cells and the formation of T-cell polarization at the contact of T cells and mast cells has been visualized by confocal laser scanning microscopy.[104] Such direct contacts of mast cells and T helper cells also render mast cells more susceptible to FcεRI-dependent degranulation.[104]

Mast cells can also influence T-cell activation by the release of exosomes. Exosomes derived from IL-4-treated mouse BMCMCs induce lymphocyte proliferation and IL-2 and IFNγ production in vitro. Injection of such mast-cell–derived exosomes, which contain costimulatory molecules (such as MHC II, CD86, CD40, CD40L, LFA-1 and intercellular adhesion molecule 1), can induce lymphocyte proliferation and cytokine production in vivo.[110]

Mast cells can enhance antigen presentation indirectly in vitro by internalizing antigen bound to FcεRI-associated IgE; this mechanism is independent of mast-cell MHC class II expression, but requires that such mast cells undergo apoptosis and then are phagocytised by other antigen-presenting cells.[111] Targeting antigens to IgE or IgG bound to mast cells can enhance the efficiency of antigen presentation,[112,113] which might be mediated by the mechanism described by Kambayashi et al,[111] or by the transfer of antigen to other APCs via mast-cell-associated exosomes.

As noted above, mouse and/or human mast cells can express many costimulatory molecules including members of the B7 family—inducible T-cell costimulator ligand (ICOSL), PD-L1, PD-L2, CD80 (also known as B7.1) and CD86 (also known as B7.2)—members of TNF–TNF receptor families—OX40, CD153, CD95, 4-1BB and glucocorticoid-induced TNF-receptor-related protein (GITR)—and CD28 and CD40 ligand (CD40L).[12,114] Moreover, mast cells can exhibit costimulatory function in vitro. For example, engagement of the costimulatory ligand OX40L expressed by human[114] or mouse[115]

mast cells and OX40 expressed by T cells is required for optimal mast-cell-dependent enhancement of T-cell proliferation[114] or cytokine production.[115]

Many secreted products of mast cells can influence T-cell activation. For example, histamine, LTB_4, PGD_2 and TNF can promote the migration, recruitment, maturation and activation of lymphocytes; and PGD_2 can enhance cytokine production by T_H2 cells.[116] Histamine promotes T_H1-cell activation through H1 receptors but conversely can suppress both T_H1 and T_H2 cell activation through H2 receptors.[117] Mast cells are also sources of many cytokines, such as IL-2, IL-4, IL-6, IL-10, IL-12, IL-13 and transforming growth factor-β (TGF-β), that can influence the polarization of naïve T cells toward T_H1, T_H2, T_H17 and T_{Reg} cells[12] and can modulate the function of distinct T-cell subsets.

Taken together, mast cells can potentially modulate T-cell-dependent acquired immunity by antigen presentation to T cells or potentiate the efficiency of antigen presentation by other APCs. Mast cells can enhance T-cell function by direct cell-cell contact, by exosome-mediated effects and/or by their secreted products. Although there is not yet clear evidence that native populations of mast cells can perform these functions in vivo, migration of mast cells to draining LN has been shown in many innate and acquired immune responses, including CHS,[118] UVB irradiation,[119] EAE,[120] cutaneous infection with *Leishmania major*,[105] injection of LPS[105] and in a model of anti-GBM (glomerular basement membrane) induced glomerulonephritis.[121] These observations suggest that mast cells derived from sites of antigen challenge have the potential to influence T-cell function within LNs, as well as at the sites in which such mast cells ordinarily reside.

Effects on CD8 T Cells

Several recent studies have specifically examined the regulation of CD8 T-cell function by mast cells. Mouse BMCMCs can stimulate bacterial antigen–specific CD8 T-cell activation by presenting bacterial antigens through MHC I.[102] A recent study by Stelekati et al confirmed that BMCMCs can present antigen to CD8 T cells in an MHC I-restricted manner, resulting in IL-2, IFNγ and MIP-1α production and can promote CD8 T-cell degranulation and cytotoxicity.[103] Intraperitoneal injection of Poly(I:C), a TLR3 ligand, induces CD8 T-cell recruitment and promotes the expression of MHC II, CD80, CD28 and complement receptors by PMC.[67] Heib et al found that mast-cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice exhibited an impaired peptide-specific cytotoxic T-lymphocyte response after transcutaneous peptide immunization together with TLR7 ligands (used as adjuvants) and that, in mice containing mast cells, treatment with ligands for TLR7 induced mast cell IL-1-dependent LC emigration and mast cell TNF-dependent LN hypertrophy.[56]

Effects on B-Cells and Immunoglobulin (Ig) Production

Several mast cell products, including IL-4, IL-5, IL-6, IL-13, CD40L and rat mast-cell protease I can influence B-cell development and function, including IgE production.[122,123] Using a coculture system, Merluzzi and colleagues recently showed that BMCMCs can promote the survival and activation of naïve B cells and promote the proliferation and differentiation of activated B cells toward IgA secreting plasma cells.[124] These effects of mast cells require both cell-cell contact and mast-cell-derived IL-6.[124] Mast-cell-derived exosomes, by effects on DC maturation and APC efficiency, can elicit specific antibody production in naive mice in the absence of conventional adjuvants.[86] In a mouse model of urinary tract infection with uropathogenic *E. coli* in WT mice, $Kit^{W\text{-}sh/W\text{-}sh}$ mice and

Kit$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT or TNF$^{-/-}$ mast cells, mast cells and mast cell TNF were found to promote a protective humoral response against *E.coli* infection.[62] Furthermore, McLachlan et al showed that antigen vaccination administrated with mast-cell-activating compounds greatly enhanced the production of antigen specific IgG (with s.c. injection) and IgA (with intranasal exposure) and the enhanced production of Ig was correlated with DC and lymphocyte recruitment to the LN and with enhanced resistance to vaccinia virus infection.[58] However, mast cells appear to make little or no contribution to antibody production in other experimental settings, in which both naïve and antigen-challenged mast-cell-deficient mice exhibit normal levels of antibodies.[7]

IMMUNOMODULATORY FUNCTIONS IN VIVO

Some of the immunomodulatory functions of mast cells that have been proposed based on in vitro studies have been confirmed in vivo using mast-cell knock-in mice or mice lacking specific mast-cell-associated proteases or lacking specific protease enzymatic activity. In many of these studies, the end points assessed included the recruitment of particular immune cells, such as granulocytes, DCs or various subpopulations of lymphocytes. Many of these studies also showed that the lack of mast cells, or a specific mast-cell product, decreased the pathology associated with the immune response or impaired its effectiveness in promoting host resistance to infection (reviewed in ref.13). While most of these studies focused on the pro-inflammatory functions of mast cells, several recent studies provided compelling evidence that mast cells can also down-regulate inflammatory responses and promote tissue homeostasis in certain experimental settings.

Host Defense against Infections

Work by many groups, using both mast cell knock-in and mast-cell-associated protease-deficient mice, has shown that mast cells can modulate host resistance and survival during several examples of bacterial infections.[8,11,19,30,34,59,125-128] The beneficial role of mast cells in host defense against acute bacterial infection was first demonstrated by two seminal studies in 1996.[125,126] In the cecal ligation and puncture (CLP) model, which is considered by some investigators to be the "gold standard" mouse model of sepsis (reviewed in refs. 17,19,30,125), host resistance and survival can be enhanced by mast cells and TNF. Treatment with SCF, that increases mast cell numbers, can provide further protection in the CLP model.[129] Activation of mast cells mediated by TLR4,[66] complement receptors,[130,131] or endothelin-1 (see ref. 17) has been shown to contribute to the ability of mast cells to enhance host resistance in CLP models. On the other hand, Smad3 and IL-15 expression in mast cells function to inhibit mast cell-mediated protection against bacterial infection.[132,133] There is evidence that MCPT-2 can contribute to neutrophil recruitment and host survival during CLP, but that, in wild-type mice, mast-cell production of intracellular IL-15 limits the mast cell's ability to produce this protease in that setting.[133] Mast-cell-deficient *Kit*$^{W/Wv}$ mice engrafted with Smad3$^{-/-}$ mast cells had significantly improved survival after CLP compared to *Kit*$^{W/W\text{-}v}$ mice engrafted with wild-type mast cells, which exhibited higher production of pro-inflammatory cytokines in the peritoneal cavity.[132]

Several mast cell functions have been implicated in host defense. These include enhancement of the recruitment or function of granulocytes,[11,125-127,134]

phagocytosis-dependent bactericidal activities (reviewed in refs. 8,59,135,136) and proteolytic degradation of endogenous mediators which would otherwise be elevated to toxic levels, such as endothelin-1 (see ref. 17) and neurotensin.[19] Secretion of proteases/enzymes and an antimicrobial peptide[137] and formation of extracellular traps that contain antimicrobial peptides, histone, DNA and tryptase,[138] have also been proposed as potential mechanisms by which mast cells exert protective functions. For example, mast-cell-derived proteases can promote host defense following intraperitoneal injection of the bacteria *Klebsiella pneumoniae* and TPSB2-deficient mice have both decreased neutrophil recruitment into the peritoneal cavity and significantly increased mortality.[34] Mast cells can also contribute to host defense by modulating acquired immunity to pathogens. They can process bacterial antigen and present it to T cells,[102] recruit lymphocytes and induce LN hyperplasia by TNF production.[139] Mast cells and mast cell-TNF have been shown to recruit T cells, DCs and other inflammatory cells into inflamed tissues and the draining LN after *E. coli* infection[62] and mosquito bites.[140] Kunder et al have reported that one mechanism by which mast cells regulate lymphocyte recruitment is by the delivery of an inflammatory signal, consisting of exocytosed heparin-containing cytoplasmic granules that also contain other mast cell mediators, particularly TNF, to draining LNs via lymphatic vessels.[60]

Such observations support the conclusion that mast cells can have important sentinel and effector roles during bacterial infection, which help to promote clearance of the bacteria, protect the host from pathology and enhance survival. However, there is evidence from work in mast-cell-engrafted C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that mast-cell-dipeptidyl peptidase-I can have effects in a severe model of CLP that decrease levels of IL-6 and reduce survival.[141] Moreover, another study in mast-cell-engrafted C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice indicates that in certain severe bacterial infections, including a model of severe CLP, mast cell production of TNF (and perhaps other mast cell functions) can exacerbate inflammation and mortality.[30] Another setting in which mast cell responses to bacteria may contribute to pathology is in atopic dermatitis, an allergic disorder in which the majority of patients have colonization of the skin with *Staphylococcus aureus*, a source of peptidoglycan which could mediate TLR2-dependent activation of mast cell cytokine production.[59,142] Considerations such as these suggest that, depending on the setting, including the severity and/or type of infection or the presence of another disorder, mast cells can either promote health or increase pathology during host responses to bacteria. Intrinsic properties of the mast cell, such as genetic predispositions to produce larger or smaller amounts of TNF and other cytokines, or the presence of other abnormalities in the host (e.g., C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice have increased numbers of neutrophils),[28-30] also may influence whether the role of mast cells in particular bacterial infections are beneficial or harmful.

In addition to their role in bacterial infections, mast cells can promote host resistance to certain parasite infections. However, the mechanisms involved have not been fully defined and may be complex, involving both local and systemic mast-cell-dependent effects (reviewed in refs. 6,8,143,144). For example, a deficiency in TPSB2 (or in IgE) was associated with markedly reduced recruitment of eosinophils to the sites of larvae deposition in skeletal muscle during the chronic phase of *Trichinella spiralis* infection, but was not associated with a detectable abnormality in the intestinal expulsion of the parasite.[144] In a model of cutaneous parasite infection by *Leishmania major*, mast cells contributed to the control of skin lesions by priming antigen-specific T cells and enhancing the recruitment of pro-inflammatory neutrophils, macrophages and DCs. Interestingly,

local mast cell activation at the infected sites is sufficient for the induction of systemic protection in this model.[143]

There is evidence that mast cells participate in host responses to certain viruses, but their precise roles in such settings are not yet clear.[8,145] Rodent[11,59,67,134] and human[11,63,146] mast cells express TLRs (e.g., TLR3) which can be activated by viral double-stranded RNA to release various chemokines and cytokines, including interferon-α and $-\beta$.[63] Some of these mast-cell-derived products may contribute to host defense against viruses.[8,11,63,67] Co-stimulation of rodent and human mast cell populations in vitro via the FcεRI and certain TLRs can enhance the cells' secretion of various pro-inflammatory mediators, suggesting one mechanism whereby bacterial or viral infections might exacerbate allergic diseases and other IgE- and mast-cell-associated disorders in vivo.[59,147,148]

Innate Responses to UVB Irradiation

Mast cells have been implicated in ultraviolet (UV)-B-induced immunosuppression and many mast-cell-associated mediators (histamine, PGE_2, serotonin, PAF, TNF, IL-4, IL-10) are produced in response to UVB irradiation (wavelengths: 280-320 nm) of the skin.[119,149] Mast cells seem to play two roles during UVB-induced inflammation. ET-1, a mediator produced upon UVB irradiation, activates mast cells to potentiate skin inflammation following a single exposure to UVB.[150] By contrast, mast cells are anti-inflammatory in a model of chronic low-dose UVB-irradiation,[24,151] at least in part by their ability to produce IL-10 and perhaps other anti-inflammatory cytokines. After a series of exposures to UVB, mast cells limited multiple aspects of the inflammatory responses and tissue pathology, including numbers of granulocytes, macrophages and T cells at the reaction sites, as well as the local tissue swelling, epidermal hyperplasia and epidermal necrosis.[24,151]

Hart et al[23] showed that the ability of a single high dose of UVB irradiation of the skin to induce systemic immunosuppression of contact hypersensitivity (CHS) responses to the hapten 2,4,6-trinitrochlorobenzene was markedly reduced in (C57BL/6 x DBA/2) F_1-*Kit*$^{W\text{-}f/W\text{-}f}$ mice but was restored following mast-cell engraftment. Several lines of evidence suggested that histamine was a major mediator of this UVB-induced, mast-cell-dependent effect.[149] Mast cells were probably also responsible for the finding that UVB irradiation suppressed delayed-type hypersensitivity (DTH) responses to allogeneic spleen cells in *Kit*$^{W\text{-}f/+}$ mice (which contain dermal mast cells) but not in *Kit*$^{W\text{-}f/W\text{-}f}$ mice (which do not contain dermal mast cells). Alard et al showed that mast cells, probably via the secretion of TNF, are required for local UVB-induced immune suppression.[152] CXCR4 dependent migration of mast cells to the LN upon UVB treatment is critical for the UVB-induced immune suppression.[119] More recently, Biggs et al provided evidence that activation of mast cells through their vitamin D receptors (VDRs) by physiologically active vitamin D_3 (i.e., 1α,25-dihydoxyvitamin D_3) is required for optimal release of mast-cell-derived IL-10, which in turn contributes to the mast cell's ability to suppress inflammation and skin pathology at sites of chronic low-dose UVB irradiation (Fig. 1).[151]

T-Cell-Dependent Responses

EAE

In the mouse model of experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG), mast cells can increase the incidence

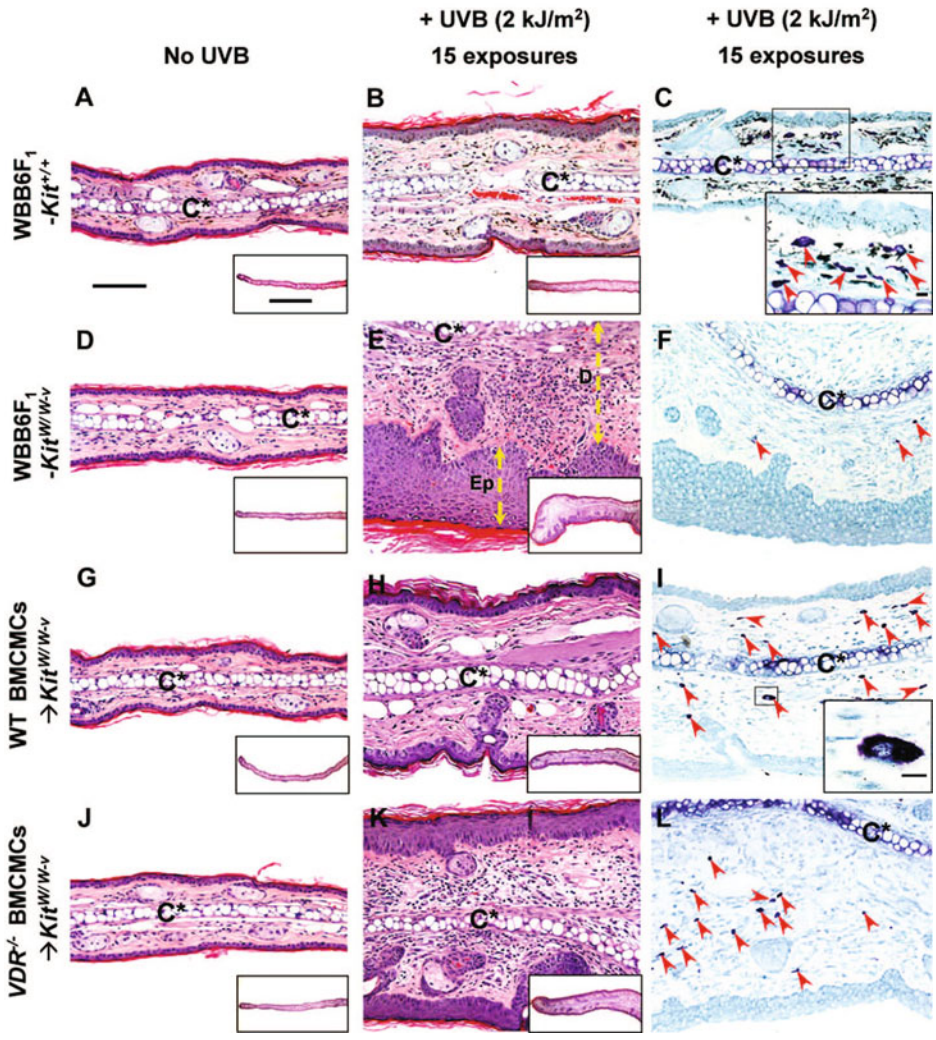

Figure 1. Mast cells and mast cell VDR expression are required to limit pathology induced by chronic low-dose UVB irradiation of ear skin. Cross-sections of ears obtained from WBB6F$_1$-*Kit*$^{+/+}$ (wild-type) mice (A-C), WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ (*Kit*$^{W/W\text{-}v}$) mice (D-F), WT BMCMC→*Kit*$^{W/W\text{-}v}$ mice (G-I), or *VDR*$^{-/-}$ BMCMC→*Kit*$^{W/W\text{-}v}$ mice (J-L) at 24 h after the final exposure to 2 kJ/m^2 UVB (15 exposures, 2 d apart; B,C,E,F,H,I,K,L); sections stained with haematoxylin and eosin (A,B,D,E,G,H,J,K), or toluidine blue (C,F,I,L). Mice that did not receive UVB irradiation were also killed for analysis of skin histology at the end of the experiment (A,D,G,J). C*, cartilage. Double-headed arrows indicate thickness of dermis (D) or epidermis (Ep). Red arrowheads indicate mast cells (C,F,I,L). Scale bars in A = 100 μm (or 1000 μm in inset) (for A,B,D,E,G,H,J,L inset), or 10 μm (in C,I insets). Results are representative of those obtained in the 3 different experiments performed, each with 3-4 mice analyzed per group. Reproduced with permission from Biggs L et al. J Exp Med 2010; doi:10.1084/jem.20091725.

and severity of the disorder.[103,153,154] Remarkably, it appears that mast cells do not have to be within the CNS to exert at least some of their important effects in MOG-induced EAE. Therefore, although systemic engraftment of mast-cell-deficient *Kit*$^{W/W\text{-}v}$ mice with

in vitro-derived BMCMCs does not result in the appearance of mast cells in the CNS of these mice, they exhibit a CNS disease severity that is similar to that in wild-type mice. Two mechanisms have been proposed to explain how extra-CNS mast cells can influence autoimmune responses to MOG. One mechanism is through the production of IL-4 in the LNs which enhances the development of encephalogenic T_H1 cells.[155] Mast cell antigen presentation that enhances the priming of MOG specific CD8 T cells has recently been shown to also contribute importantly to EAE pathogenesis.[103] More recently, Brown's group reported that, in this model of EAE, mast cells in the meninges of the brain can enhance blood-brain-barrier permeability and local inflammatory cell infiltration to CNS, including the recruitment of neutrophils by mast cell-derived TNF.[156]

Mouse Models of Asthma

Various mouse models have been used to elucidate the roles of mast cells in allergic airway inflammation. Depending on the mode of allergen sensitization, the contribution of mast cells is most obvious when the mice are sensitized and/or challenged with low doses of antigens, with adjuvant-free antigen, or sensitized with antigen by inhalation route (reviewed in refs. 157,158). In mouse models of asthma in which mast cells enhance the response, mast cells[158-163] and mast-cell-derived TNF significantly contribute to both airway hyperreactivity and airway inflammation.[159,158,160-163] Some of the effects of TNF probably reflect its ability to promote T-cell recruitment and T_H2-type cytokine production.[161] Another mechanism which may contribute to "mast-cell-dependent" effects on T cells during models of allergic inflammation is FcεRI-dependent mast cell secretion of LTB_4, with subsequent recruitment of effector CD8 T and CD4 T cells to sites of airway inflammation.[164-166] Mast cells also contribute to a model of T_H17-cell-dependent, neutrophil-associated lung inflammation in ovalbumin (OVA)-challenged, OVA-specific T-cell receptor transgenic mice.[167]

Moreover, in a mouse model of chronic allergic inflammation of the airways, mast cells are required for the full development of several features of tissue remodeling, including increased numbers of mucus-producing goblet cells in the airway epithelium and increased lung collagen deposition, changes accompanied by a mast-cell-dependent exacerbation of airway hyperreactivity to methacholine.[158] In this mouse model of chronic asthma, mast cell activation mediated by antibody dependent- and independent-mechanisms contributes to the full manifestation of the airway disease.[158] Finally, in one model of allergic inflammation of the airways, mice lacking the mast cell chymase, MCPT-4, compared to the corresponding wild-type mice, exhibited more substantial increases in airway hyperreactivity to methacholine, increased airway inflammation and thickening of bronchial smooth muscle.[168] These findings indicate that at least one mast cell product may help to limit the pathology associated with allergic inflammation.

The Two Faces of Mast Cells in CHS

Under some experimental conditions, mast cells are required for the optimal elicitation of the inflammation associated with mouse models of hapten-induced CHS (reviewed in ref. 55). Mast cells and IgE can contribute to LC emigration and promote effective sensitization to the chemical hapten oxazolone,[87] as well as contribute to the effector phase of the responses, including the elongation of cutaneous nerve fibers.[169] However, mast cells can also have negative immunomodulatory functions in some models of CHS. Mast cells markedly limited the magnitude and duration as well as promoted the resolution, of

CHS responses induced in mice by the hapten 2,4-dinitro-1-fluorobenzene) (DNFB) or by urushiol, which is the hapten-containing sap of poison ivy or poison oak.[24] In these responses, mast cells substantially inhibited the development of pathology, including the leukocyte infiltration, epidermal hyperplasia and epidermal necrosis associated with these responses. Several lines of evidence indicated that mast cell production of IL-10, mediated at least in part by antigen-specific IgG1 binding to FcγRIII on mast cells, is essential for mast cell anti-inflammatory function in these responses.[24] Although the pathways that link mast-cell-derived IL-10 (or other mast cell mediators that are relevant in this setting) to the observed tissue changes remain to be defined, mast cells and mast-cell-derived IL-10 may influence these responses through a complex combination of direct and indirect effector and immunoregulatory functions.

In another study, the nature of the mast cells contribution to Ox-induced CHS was found to depend on the dose of hapten used to immunize the mice. Norman et al showed that mast-cell-deficient $Kit^{W/W\text{-}v}$ mice, when compared to wild-type mice, exhibited reduced CHS responses (associated with little or no local production of IL-10) when immunized with low doses of Ox but more severe reactions (associated with local production of IL-10) when immunized with high doses of Ox. This study suggests that, depending on the strength of immunization, mast cells can regulate the magnitude of the T-cell-associated CHS responses by altering the cytokine microenvironment.[170]

Antibody-Dependent Responses

Mast cells can contribute substantially to the disease pathology induced by auto-antibodies. In a mouse model of anti-glucose-6-phosphate isomerase (GPI)-induced destructive arthritis, work in $Kit^{W/W\text{-}v}$ mice indicated that mast cells can contribute to the initiation of joint inflammation by FcγR-dependent release of IL-1.[171,172] $Kit^{W/W\text{-}v}$ mice also did not develop wild-type levels of joint swelling in a model of arthritis elicited by injection of antitype II collagen mAbs followed by LPS. By contrast, $Kit^{W\text{-}sh/Wsh}$ mice developed features of arthritis in this model that were similar to those of the corresponding wild-type mice.[29] The differences in the susceptibility of these two strains of mast-cell-deficient mice to the autoantibody-induced joint disease may be due to the numbers of neutrophils in these animals. $Kit^{W/W\text{-}v}$ mice have reduced numbers of bone-marrow and blood neutrophils, whereas $Kit^{W\text{-}sh/W\text{-}sh}$ mice have increased numbers of bone-marrow and blood neutrophils (which may diminish the importance of any contribution of mast cells in this strain). Mast cells have been shown to recruit neutrophils in a mouse model of bullous pemphigoid (an autoimmune disease of the skin) elicited by injection of anti-hemidesmosomal IgG.[173]

On the other hand, mast cells can exert anti-inflammatory and protective functions in some models of antibody-mediated diseases. In a model of experimental anti-GBN glomerulonephritis, $Kit^{W/W\text{-}v}$ mice developed more severe glomerular damage, with more intense T-cell and macrophage infiltration, as well as more severe proteinuria and higher mortality, than wild-type mice or mast cell-engrafted $Kit^{W/W\text{-}v}$ mice.[121] Although mast cells are not detectable in the kidneys after induction of this model of glomerulonephritis, mast cell tryptase (mouse transmembrane tryptase [mTMT]) was detected in the draining LNs. These results suggest that whatever anti-inflammatory functions mast cells exert in this model reflect the actions of mast cells in draining LNs and/or other sites distant from the organ-related pathology, not mast cells at the site of inflammation.[121] Moreover, there is evidence that at least one mast-cell-derived product, MCPT-4, can promote pathology in this model,[174] suggesting that mast cells can have complex roles in this and perhaps

other immune responses, with some of their mediators having pro-inflammatory effects and others having anti-inflammatory functions.

OTHER NEGATIVE IMMUNOMODULATORY FUNCTIONS OF MAST CELLS

Mast cells also contribute to other models of immunosuppression. Depinay et al[175] reported that the bites of *Anopheles* mosquitoes can impair the development of antigen-specific T-cell responses in a model of DTH to ovalbumin in mice, but only if mast cells are present in the bitten skin. How mast cells mediate immunosuppressive function in this model remains to be elucidated. Limon-Flores et al[176] used $Kit^{W\text{-}sh/W\text{-}sh}$ mice to show that PGE_2 production by mast cells and mast cell migration to draining LNs, were critical for observing the mast-cell-mediated inhibition of CHS responses elicited by epicutaneous application of jet fuel to mouse skin.[176] IL-10 also has been implicated as contributing to immunosuppression in this model.[176] However, while the jet fuel-induced immunosuppression of CHS in $Kit^{W\text{-}sh/W\text{-}sh}$ mice containing IL-10-deficient skin mast cells was greater than that seen in mast-cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice, it was not as substantial as that in wild-type mice or in $Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with wild-type mast cells.

In addition to determining whether mast cells are important sources of IL-10 or PGE_2 (or other factors that can mediate immunosuppressive effects) in other examples of immunosuppression, it will be of interest to assess whether mast cells and mast-cell-derived products have important effects on T_{Reg} cell numbers, phenotype and/or function in models of CHS or chronic UVB irradiation, or in any of the many other settings in which T_{Reg} cells are thought to have an important role.[177,178]

Studies that investigate the potential interactions between mast cells and T_{Reg} are of great interest in helping to unravel the mechanisms of mast-cell-dependent immunosuppression.[179,180] The first in vivo study of this type was that of Lu et al,[109] who showed that mast cells were essential for the optimal induction of peripheral tolerance to skin allografts, which requires the participation of $CD4^+CD25^+FoxP3^+$ T_{Reg} cells. T_{Reg} cells are a source of IL-9 and IL-9 can mediate the suppression of alloreactive CD8 T cells and act as a mast-cell survival and/or growth factor that can enhance mast-cell function (reviewed in ref. 109). Local production of IL-9 (by T_{Reg} cells and/or other sources) may have contributed to the development and perhaps influenced the function, of mast cell populations within the tolerant allografts.

Consistent with the observation that mast cells can promote peripheral tolerance to skin allografts in mice, evidence derived from studies of c-*kit* mutant $Kit^{Ws/Ws}$ versus wild-type rats (in which grafts from the non-inbred $Kit^{Ws/Ws}$ and wild-type rats were transplanted into $Kit^{Ws/Ws}$ and wild-type rats, respectively) suggests that mast cells may favor the survival of heterotopic cardiac grafts in rats.[181] However, work in C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ versus wild-type mice indicates that the presence or absence of mast cells in the recipient mice (which received cardiac allografts derived from other, mast-cell-containing, mouse strains) makes little or no difference in the features of either an acute or chronic model of cardiac allograft rejection.[182] Moreover, local or systemic mast cell degranulation can impair T_{Reg} function and lead to the T-cell-dependent acute rejection of established tolerant skin allografts.[183] The latter finding is in accord with the results of an in vitro coculture study showing that mast cells can inhibit T_{Reg} suppression, but promote T effector cell expansion and T_H17 cell differentiation via an IL-6 and cell proximity dependent

(OX40/X40L) manner.[184] Taken together, the results of both in vivo and in vitro data suggest that multiple factors may be able to influence whether mast cells have positive, negative, or neutral effects on allograft survival.

In mice, mast cell IgE-dependent effector function can be modulated by T_{Reg}. In a mouse model of IgE-mediated passive systemic anaphylaxis, assessment of histamine levels in the serum showed that mast cell activation in response to challenge with IgE and specific antigen was significantly increased, relative to values in wild-type mice, either in wild-type mice that had been depleted of T_{Reg} in vivo or in OX40-deficient mice.[185] In vitro studies showed that T_{Reg} can directly inhibit FcεRI-dependent mast cell degranulation (but not mast cell production of IL-6 or TNF) through cell–cell contact involving interactions between OX40 expressed on T_{Reg} and OX40 ligand expressed by mast cells.[185] This study defined a novel, T_{Reg} -dependent mechanism, which can suppress mast cell degranulation and which could serve to limit anaphylaxis and perhaps other IgE-dependent responses.

CONCLUSION

In summary, mast cells can exert positive immunoregulatory functions in vivo that can either enhance host defense or promote disease, that reflect actions of the mast cell's stored mediators and/or cytokines and that can be mediated by functions of mast cells that reside either at the site of the immune response or at peripheral sites, such as within LNs. As outlined above, mast cells can also have negative immunomodulatory functions, in addition to their well-established roles as effector cells. Understanding in detail how individual positive or negative immunomodulatory functions can be induced or suppressed in various mast cell populations will continue to be of considerable interest.

However, understanding the specific roles of mast cells in immunomodulation during particular immune responses in vivo may be quite challenging. For example, it already is clear that mast cells can have either positive or negative immunomodulatory functions in what would appear to be very similar settings, such as in different mouse models of CHS.[7,10,87,186,187] One may even speculate that immune responses will be found in which mast cells first promote the sensitization phase of the response, then help to initiate the local inflammation that occurs when the host subsequently is exposed to specific antigen and finally help to limit the extent of and/or resolve, the ensuing inflammation and associated tissue pathology.

In support of this hypothesis, both in vitro and in vivo data strongly suggest that one of the mechanisms that promote the mast-cell-dependent IL-10 production that in turn limits certain CHS responses is the activation of mast cells by immune complexes of specific antigen and IgG1.[24] These antigen-specific IgG1 antibodies develop, probably by mast cell-independent mechanisms, in response to the initial exposure to hapten during the sensitization phase of CHS.[24] Thus, in this model, the development of an aspect of the humoral component of the immune response to hapten challenge (i.e., antigen-specific IgG1) results in the generation of a signal (i.e., antigen-IgG1 immune complexes) that promotes an anti-inflammatory phenotype in the mast cells resident at the site of the local reaction.

The notion that mast cells might first promote the sensitization and/or elicitation phases of an immune response and then help to limit or resolve the local tissue alterations induced by antigen challenge, is consistent with the hypothesis that one key function of

this cell type is to promote homeostasis—even in those instances when the mast cells also have a major role in perturbing homeostasis in order to promote host defense.[17-20]

It will be of great interest to define in detail how and under which circumstances, the positive and negative immunomodulatory functions of mast cells can significantly influence the development, magnitude or kinetics of innate or acquired immune responses. It will also be of interest to assess whether such mast-cell functions can be manipulated to achieve therapeutic ends, such as the enhancement of immune responses that promote health or the suppression of those that result in disease.

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CHAPTER 12

MAST CELL PROTEASES AS PROTECTIVE AND INFLAMMATORY MEDIATORS

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Abstract: Proteases are the most abundant class of proteins produced by mast cells. Many of these are stored in membrane-enclosed intracellular granules until liberated by degranulating stimuli, which include cross-linking of high affinity IgE receptor $F_c\varepsilon RI$ by IgE bound to multivalent allergen. Understanding and separating the functions of the proteases is important because expression differs among mast cells in different tissue locations. Differences between laboratory animals and humans in protease expression also influence the degree of confidence with which results obtained in animal models of mast cell function can be extrapolated to humans. The inflammatory potential of mast cell proteases was the first aspect of their biology to be explored and has received the most attention, in part because some of them, notably tryptases and chymases, are biomarkers of local and systemic mast cell degranulation and anaphylaxis. Although some of the proteases indeed augment allergic inflammation and are potential targets for inhibition to treat asthma and related allergic disorders, they are protective and even anti-inflammatory in some settings. For example, mast cell tryptases may protect from serious bacterial lung infections and may limit the "rubor" component of inflammation caused by vasodilating neuropeptides in the skin. Chymases help to maintain intestinal barrier function and to expel parasitic worms and may support blood pressure during anaphylaxis by generating angiotensin II. In other life-or-death examples, carboxypeptidase A3 and other mast cell peptidases limit systemic toxicity of endogenous peptides like endothelin and neurotensin during septic peritonitis and inactivate venom-associated peptides. On the other hand, mast cell peptidase-mediated destruction of protective cytokines, like IL-6, can enhance mortality from sepsis. Peptidases released from mast cells also influence nonmast cell proteases, such as by activating matrix metalloproteinase cascades, which are important in responses to infection and resolution of tissue injury. Overall, mast cell proteases have a variety of roles, inflammatory and anti-inflammatory, protective and deleterious, in keeping with the increasingly well-appreciated contributions of mast cells in allergy, tissue homeostasis and innate immunity.

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INTRODUCTION

Several recent reviews provide in-depth coverage of particulars of mast cell protease biochemistry, genetics and biological function.[1-4] The present chapter emphasizes mast cell protease function as it relates to host defense and its frequent by-product, inflammation. The link between mast cell proteases and inflammation is not (as some might assume) automatic; rather, the overall effect can be anti-inflammatory and homeostatic. This chapter cannot do justice to all of the work being done in this field, which has expanded rapidly in the past five years, but it will summarize major recent findings shaping current notions of protease contributions to mast cell function and pathobiology.

PROTECTIVE AND ANTI-INFLAMMATORY EFFECTS

Control of Neurogenic Inflammation: Tryptases, Calcitonin Gene-Related Peptide and the Triple Response of Lewis

A classical response to injury (such as a that created by stroking skin with a blunt instrument) is a red line appearing at the site of injury, followed by transient flare or redness surrounding the region of injury and a wheal due to edema at and near the site injury. This is the so-called triple response of Lewis. The redness or "rubor" aspect is a cardinal sign of inflammation, along with "calor" (heat) and tumor (swelling). The red line is partly due to the release of histamine from mast cells under the influence of the neuropeptide substance P discharged from sensory nerves stimulated by the injury. The flare is attributed mainly to release of calcitonin gene-related peptide (CGRP) from sensory nerves stimulated by antidromic propagation of sensory nerve signals originating at the site of injury. The transience of CGRP-induced vasodilation is thought to be due to extracellular release of peptidases (Table 1) from mast cells activated by substance P.[5] CGRP is hydrolyzed by tryptases and, kinetically speaking, may be the best natural substrate yet identified for human β-tryptase,[6] which inactivates CGRP's vasodilating actions.[7] More recent evidence suggests that primary spinal afferent neurons containing CGRP and substance P also contain protease-activated receptor (PAR)-2,[8] which can be activated by tryptases.[9-14] Although tryptase is weak compared to trypsin as a PAR-2 activator, mast cells are often near neurons,[15] which may be exposed to high concentrations of tryptase during mast cell degranulation. Thus nerves, substance P, CGRP and tryptases may be involved in feedback loops that limit neurogenic inflammation. In effect, tryptases detoxify CGRP, which is perhaps the first-described example of a more general function for tryptases discussed below in connection with venoms and toxic nonneural peptides.

Thresholds for Protective Nociception and Bronchoconstriction: Roles of Tryptases and PAR-2

Tryptase-activated neural PAR-2 is implicated in the component of itching in atopic dermatitis that is unresponsive to antihistamines.[16,17] Furthermore, a recently appreciated phenotype of PAR-2-null mice is lowered nociceptive thresholds, such as

Table 1. Comparison of some mast cell proteases in mice and humans

Protease Class	Human	Mouse
SERINE		
Tryptase-like		
Active:	βI, βII, βIII, γ	mMCP-6, -7*, γ; mastin/mMCP-11**
Inactive:	α, δ, $βIII^{FS}$; mastin	mMCP-7*
Chymase-like		
Active:	CMA-1/α; Cathepsin G	mMCP-1, -4, -5***; Cathepsin G
Inactive:		mMCP-2
Plasminogen activator	t-PA	?
CYSTEINE/THIOL		
Cathepsins	DPPI/C, ?others	DPPI/C, B, L, S
METALLO		
Carboxypeptidase	CPA3	CPA3
Matrix metallo	MMP-9	MMP-9
ADAM, other	?	ADAM17, neurolysin
ASPARTYL	Renin	Renin

* not expressed in some strains of laboratory mice
** expressed primarily in basophils
*** elastolytic, not chymotryptic

those involving sensitivity to dermal and visceral pain,[18] although a role for tryptases in setting pain thresholds in vivo is partly speculative at this point. Itch and pain are both essentially protective, because they alert the host to the presence of pathogens or impending tissue damage. PAR-2 also is expressed in nonneural tissues and cells, such as airway smooth muscle;[11] indeed, isolated bronchi constrict in response to PAR-2 agonists, including tryptases.[19-22] Nonetheless, it is not yet clear that the bronchoconstricting activities of tryptases are mediated fully via interactions with PAR-2, especially since most studies find that tryptase, rather than constricting smooth muscle on its own, potentiates the actions of known constrictors, such as histamine, so that they act at lower concentrations and to greater maximum effect.[19,20,22,23] Furthermore, PAR-2 agonists acting on epithelium rather than smooth muscle can cause bronchodilation by stimulating epithelial release of PGE_2.[24] Alternative mechanisms by which tryptase may promote bronchoconstriction include degrading bronchodilating peptides (leaving bronchoconstrictors unopposed),[6,25-27] spreading degranulation signals,[23] untethering muscle from the bronchial wall by cleaving extracellular matrix or activating matrix-cleaving proteases[28] and desensitizing smooth muscle cells to bronchorelaxing β-adrenergic agonists.[29] Tryptase's overall effect is to promote bronchial hyperresponsiveness, a hallmark of asthma. In essence, this is protective, if one accepts that a major purpose of airway smooth muscle is to guard airways from entry of unwanted flora, fauna and other noxious substances, for which mast cells can be sentinels. Surprisingly, the actual function and true benefits of airway smooth muscle are unknown. Clearly, the purpose is not to cause asthma.

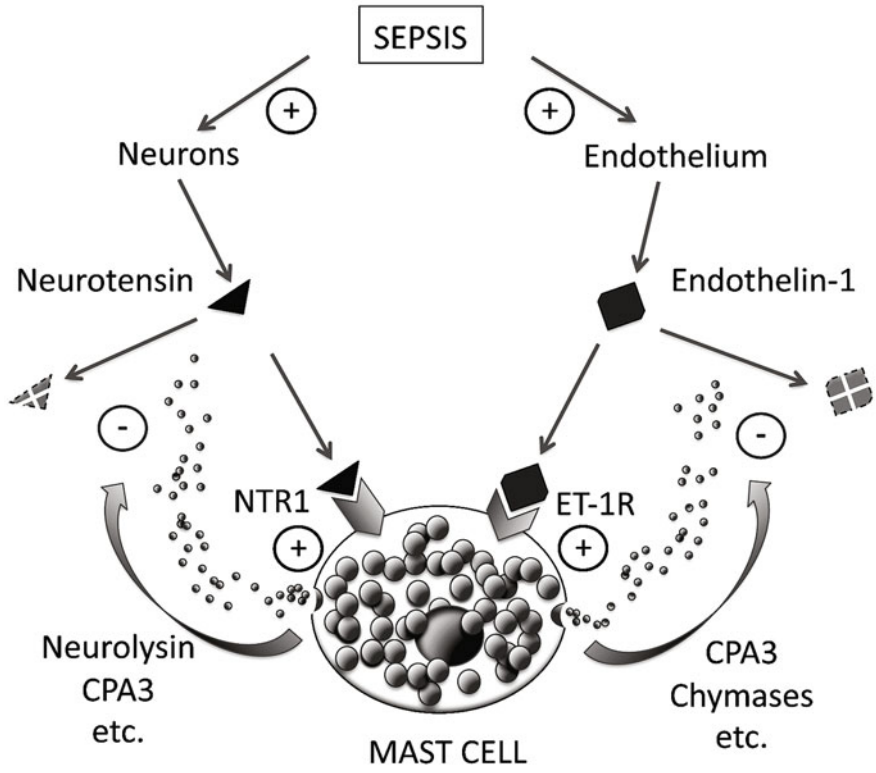

Figure 1. Mast cell-mediated protection from toxic endogenous peptides in sepsis. Bacterial sepsis is associated with release of toxic peptides like neurotensin and endothelin-1 from nerves and vascular endothelium, respectively. These peptides are recognized by receptors on mast cells, stimulating release, activation or upregulation of detoxifying peptidases, such as carboxypeptidase A3 (CPA3), chymases and neurolysin. This is an example of a homeostatic function of mast cells.

Feedback Detoxification of Neurotensin by Neurolysin and Carboxypeptidase

Neurotensin is a 13-residue fragment of a much larger polypeptide that includes the neuromedin N. Neurotensin gains partial protection from degradation by posttranslational cyclization of its N-terminal amino acid to pyro-glutamate, thereby eliminating its free amino terminus and reducing its sensitivity to shortening by amino-peptidases. Neurotensin is usually classified as a neuropeptide, since it can originate from neurons. It causes hypotension when injected into mice; moreover, endogenous neurotensin can influence mortality in septic shock, because mice lacking neurotensin are less likely to survive septic peritonitis. Serum levels of neurotensin are elevated in humans with shock and sepsis. The origin of serum and peritoneal neurotensin is suspected to be neural; however, the actual sources in these conditions are not known.[30] Intriguing experiments in mice subjected to cecal ligation and puncture, which models septic peritonitis from a ruptured appendix, suggest that mast cells respond to neurotensin in the peritoneum and play a detoxifying role significant enough to affect survival. Although neurotensin is not a strong mast cell degranulator, the detoxifying effect of mast cells depends at

least partially on mast cell expression of neurotensin receptor 1, which may signal these cells to express an inactivating metallopeptidase, neurolysin—possibly on the cell surface (see Fig. 1). Secreted carboxypeptidase A3 also appears to play a role, presumably by removing residues from neurotensin's unprotected C-terminus. These findings suggest one explanation for the known benefit of peritoneal and mesenteric mast cells in recovering from cecal ligation and puncture. Whether mast cell-mediated detoxification of neurotensin is important in other types of sepsis and shock and in humans, remains to be determined.

Detoxification of Endogenous Nonneural Peptides and Proteins

Endothelin

Many endogenous peptides have the potential to harm as well as to help when produced in response to microbial invasion or tissue injury. One particularly closely examined example, intriguing both in regard to its complexity and physiological importance, is provided by endothelins, which are family of peptides produced by endothelial cells in response to injurious stimuli. Mature endothelins act by engaging receptors on the surface a variety of cells, including vascular smooth muscle and mast cells. They can have acute and longer-term effects, including vasoconstriction, mast cell activation and vascular remodeling. Drugs antagonizing interactions of endothelins with their receptors have found a place, for example, in treating idiopathic pulmonary hypertension. Maurer and colleagues[31] described an intriguing nexus between endothelin and mast cells in the peritoneum (see Fig. 1). Endothelin injected into the peritoneal cavity of mice is toxic and, in sufficient doses, lethal. It also has toxic potential when produced endogenously in the context of septic peritonitis. Mouse peritoneal mast cells possess Type A endothelin receptors and respond to endothelin by releasing destructive peptidases, which may include chymases and mast cell carboxypeptidase A3. Schneider and colleagues established more recently that removal of a single tryptophan residue from endothelin's C-terminus is the principal inactivating event[32] in mice. Although other mast cell peptidases, including mouse chymases, can nick endothelin internally, this is not necessarily inactivating, because of stabilizing disulfide bonds. Indeed, the overall contribution of chymases to endothelin homeostasis is uncertain and may be species-specific, because some chymases can process and *activate* endothelins from larger, precursor forms. For example, human chymase processes precursor "big" endothelins into a novel, bronchoconstricting form (endothelin [1-31]),[33] whereas rat chymases are less selective and degrade big endothelins as well as endothelin [1-31], which possesses bronchoconstricting, vasoconstricting and vascular smooth muscle-proliferating activity[33,34] and thus has potential to contribute to pathological bronchoconstriction in human asthma and vascular remodeling in pulmonary hypertension, systemic atherosclerosis and restenosis after angioplasty. In reference to mice specifically, it should be noted that the overall role of endothelin-1 in asthma-like bronchoconstriction is not clear, since endothelin-1-null mice exhibit airway hyper-responsiveness.[35] In humans, then, it appears that mast cells plausibly are involved in activating or inactivating endothelin (or both in succession, since activator chymase and inactivator carboxypeptidase A3 are usually but not inevitably found in the same subsets of mast cells and released together).[36-38] Thus, it is reasonable to hypothesize that the role of human mast cells activated by already-mature endothelin is to inactivate the peptide, i.e., to limit toxicity by preventing accumulation and shortening

duration of action. However, the role of human mast cells activated by other stimuli, such as allergen, first may be to activate via chymase-mediated processing of precursor big endothelins to smaller active forms, followed perhaps by mast cell carboxypeptidase A3-mediated inactivation.

Cathelicidin

In studies of human lung mast cells, Schiemann and colleagues showed that the antibacterial peptide, cathelicidin LL-37 (production of which is induced during inflammation), stimulates mast cells to degranulate and secrete β-tryptase, which in turn inactivates cathelicidin.[39] This is an example of immunomodulatory negative feedback similar to that described for endothelin. On the other hand, human chymase and cathepsin G in skin mast cells can activate platelet-derived connective tissue activating peptide III to generate neutrophil-activating peptide II, a neutrophil chemokine.[40] Therefore, downstream effects of human mast cell degranulation on inflammation will depend on the nature of mast cell stimulus, the protease phenotype of the stimulated mast cell and the local availability of targets cleavable by the secreted proteases.

Cytokines and Interleukins

The ability of mast cells to degrade cytokines can be striking. In studies of activated human skin mast cells, exocytosed proteases markedly diminish immunodetection of mast cell-secreted cytokines like IL-6, IL-13 and TNFα, presumably by destroying epitopes recognized by antibodies.[41] Inhibitor screens suggest that chymase and cathepsin G are more responsible than are tryptases. These effects are likely to be anti-inflammatory, to the extent that they reduce biological activity as well as immunoreactivity of inflammatory cytokines. Unless countered by inhibitors, this peptidase activity causes considerable underestimation of mast cell production of several cytokines. The in vivo importance of these effects is less clear, because chymases and cathepsin G are released into environments rich in inhibitors, such as α_2-macroglobulin and α_1-antichymotrypsin.[42-44] There are, however, hints from mice that modulation of cytokine activity can be biologically significant in vivo. In septic peritonitis, hydrolytic inactivation of mast cell-derived IL-6 by mMCP-6 tryptase (and perhaps other peptidases) appears to lead to increased mortality.[45] This suggests that cytokine-cleaving activities of mast cell proteases can be counterproductive, even while reducing inflammation.

Detoxification of Snake and Bee Venoms

Mast cells have a well-deserved reputation for contributing to severe anaphylactic reactions to bee and wasp stings. Numerous deaths result from systemic release of mast cell mediators of shock and inflammation. Although hymenoptera venoms are complex mixtures of peptides and proteins, some of which directly degranulate mast cells, severe reactions are mediated by mast cell-bound IgE recognizing venom components based on prior sensitization. Because of this association between mast cells and fatal reactions to envenomation, recent evidence[46] that mast cells protect mice from lethality of some venoms is at first glance counter-intuitive. One example was provided by sarafotoxins, a class of venoms produced by snakes related to mole vipers. Sarafotoxins are homologous to endothelins, which, as discussed earlier, activate mast cells to release peptidases. Metz

and colleagues[46] showed that mast cell detoxification of sarafotoxins involves similar mechanisms and affords some protection from lethal outcomes of envenomation. As is true for endothelin, carboxypeptidase A3 plays a prominent role in disarming the toxin. It is not yet clear whether this effect applies broadly to other venom peptides and proteins. However, mast cells protect mice from hypothermia and death caused by the venom of two snakes (rattlesnake and copperhead) not containing sarafotoxins. Carboxypeptidase A3 involvement in these cases is not as prominent. Thus, for some venoms, other detoxifying peptidases, like chymases and tryptases, may be important. By unclear mechanisms, mast cells also partially protect mice from effects of honeybee venom,[46] which contains mast cell-degranulating apitoxins.

Secretion, Activation and Destruction of Matrix Metalloproteinases (MMPs)

MMPs in Remodeling and Resolution of Inflammation

The classic gelatinolytic MMPs produced by inflammatory cells are MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Although mast cells and mast cell lines can secrete MMP-2 and MMP-9,[47-51] these enzymes also are produced by a variety of other inflammatory cells, which are likely to be the dominant source in inflamed tissues. MMP-9, for example, is especially abundant in neutrophils and its levels in inflamed tissues and fluids tend to track with the number of neutrophils. It is not clear whether the well-known and often-assessed capacity of these enzymes to hydrolyze denatured collagen (gelatin) in polyacrylamide gels mirrors or predicts their roles in vivo, for these enzymes cleave a variety of proteins[52,53] and there is perhaps no equivalent of gelatin in vivo. MMP-9-null mice have phenotypic abnormalities involving long bone angiogenesis,[54] but there is no apparent deficit in airway inflammatory angiogenesis and lymphangiogenesis stimulated by mycoplasma infection and these mice do develop neutrophilic pneumonia.[55] Although MMP-2 (which increases in lungs of infected MMP-9-null mice) potentially could compensate for lack of MMP-9, MMP-2/MMP-9 double knockout mice have a neutrophilic pneumonia and airway angiogenesis phenotype similar to that of MMP-9-null mice.[55] Data from mice suggest that allergic pulmonary inflammation may differ in this regard[53,56,57] because lack of MMP-2 and, especially of MMP-9, hinders *egress* of recruited eosinophils and other leukocytes from pulmonary interstitium into the airspaces, from which they would otherwise be cleared via the mucociliary and/or macrophage-mediated apoptotic pathways. This clogs interstitial spaces with leukocytes, impairs gas transfer and "asphyxiates" the mice. These findings are intriguing because they suggest that MMP-2 and MMP-9 promote resolution of allergic inflammation. Indeed, studies of other types of inflammation also suggest that control of inflammation and associated remodeling may be an important function of these MMPs, as in a model of bronchopulmonary dysplasia, in which transgenic *Mmp9* $^{-/-}$ mice have more pulmonary macrophages and alveolar hypoplasia.[58]

Mast Cells as a Source of Gelatinolytic MMPs

The most detailed evidence that mast cells produce gelatinolytic MMPs stems from studies of canine, mouse and human mast cells.[47,49,50,59] In canine mast cell lines, MMP-9 production is regulated by KIT ligand and TGFβ.[48] MMP-9 appears to be released in a constitutive and regulated manner as a pro-form bound to MMP

inhibitor tissue inhibitor of metalloproteinases (TIMP)-1. Thus, MMP-9 is secreted as an inactive, pro-MMP-9/TIMP-1 complex.[47,60] Activation of the mast cell-secreted complex occurs outside of the cell, especially in cells that are stimulated to release contents of serine protease-rich secretory granules. Because mast cell MMP-9 seems to be secreted independently of its serine protease activators, it is likely that the pro-MMP/TIMP-1 complex originates in a compartment separate from the classic secretory granule. Human mast cells also exhibit the interesting property of secreting MMP-9 upon direct contact with T-lymphocytes.[50]

Activation, Disinhibition and Destruction of MMPs by Chymases

The interactions of mast cell chymases with MMP-9 are biochemically intriguing and possibly unique. Studies of canine mast cell chymase and pro-MMP-9 reveal that chymase activates pro-MMP-9 by selectively hydrolyzing residues in the MMP-9 pro-peptide.[47,60] This yields a large gain in solution-phase proteolytic activity. Beyond this, chymase activates MMP-9 even while bound to TIMP-1[61] and is the only MMP-9 activator shown to possess this capability. Because much extracellular MMP-9 is imbedded in matrix as a TIMP-1-bound pro-enzyme, chymase released from mast cells can bring MMP-9 to life from latency and launch cascades of MMP activation initiated by MMP-9, which can activate other MMPs. Chymase induces the activation of TIMP-1-bound MMP-9 by cleaving TIMP-1 itself. Although interactions between chymase and MMP-2 have been studied less intensively, chymase inhibitors are reported to decrease intimal hyperplasia in balloon-injured canine carotid arteries, with an accompanying decrease in activated MMP-2.[62] Although the biochemistry of MMP-9 has been explored in greatest detail using canine enzymes and mast cell lines, the phenomenon occurs in other mammals. In mice, for example, the principal pro-mMMP-9-activating chymase is mMCP-4,[49,51] whose actions are hypothesized to be important in tumor growth and invasion in a model of skin cancer[49] as well as in collagenous ear thickening and lung fibronectin accumulation. These responses are attributed to reduced matrix turnover in mMCP-4-null mice,[51] which also suggests that mMCP-4 regulates levels of MMP-2.[51] More recently, a study of chymase inhibitors in a colitis model revealed markedly reduced colonic MMP-9 levels in inhibitor-treated mice.[63] Similar effects occur in angiotensin II-induced aortic aneurysms.[64] Chymase can destroy MMP-9 activity with prolonged incubations, although its preference appears to be for activating cleavages. It should be stressed that the net effect of MMP-9 activation by chymases could be anti-inflammatory, given MMP-9's involvement in resolution of inflammation.

Mast Cell Protease-Facilitated Activation of the Renin-Angiotensin System

Updating Paradigms

Research over the past two decades has built a solid body of evidence supporting physiologically important mast cell peptidase activation of the renin-angiotensin system, whose end product is the vasoactive octapeptide *angiotensin II*. Several aspects of involvement of mast cell peptidases in generating angiotensin II are at odds with the partly outmoded concept that the key steps in generating angiotensin II occur within vessels, starting with regulated release of the aspartyl peptidase renin

into the bloodstream by kidney cells responding to drops in renal perfusion pressure. According to the classical paradigm, renin then cleaves off a portion of the N-terminus of a circulating protein, angiotensinogen, to generate the decapeptide angiotensin I. This inactive peptide is then hydrolyzed by angiotensin converting enzyme (ACE), an ecto-metallopeptidase on the surface of pulmonary vascular endothelial cells, to generate the angiotensin II, which binds to receptors in arteriolar smooth muscle to constrict vessels and acts on the adrenal gland and kidney to promote retention of sodium and water. This process is homeostatic, being designed to preserve blood pressure. Additional essentially protective roles may include vasoconstriction to reduce bleeding and edema during tissue injury. In this regard it is interesting to note the association between use of ACE inhibitors and more severe systemic reactions to insect stings.[65] ACE and angiotensin II are unquestionably important in human health, as shown by the success of therapeutic agents directed against ACE or receptors of its product, angiotensin II. As discussed in more detail below, mast cells complicate the classical paradigm by providing the means of generating both angiotensin I and II *outside* of vessels, using pathways that neither involve ACE nor are blocked by ACE inhibitors. Because mast cells outside of the kidney can be a source of renin, they can also initiate angiotensin production in a variety of tissues in response to events unconnected to regulation of blood pressure and volume. Recent research on angiotensins also suggests that angiotensin II is a trophic factor with chronic affects on growth and remodeling of many tissues and that angiotensin II-inactivating peptidases may be important in regulating angiotensin activity. Furthermore, studies suggest that there may be "good" and "bad" receptors for angiotensin II in the context, for example, of responses to lung injury.[66,67]

Generation of Angiotensin II by Chymases and Cathepsin G

The idea that some mast peptidases generate bioactive angiotensin II is not new. Travis, Wintroub and others showed that human mast cell chymase and cathepsin G process angiotensin I into angiotensin II in vitro more than a quarter of a century ago,[68,69] not long after the enzymes were first purified. Later, convergence of work in different laboratories culminated in a realization that a potent, non-ACE, angiotensin II-generating peptidase extractable from human heart tissue is, in fact, indistinguishable from human mast cell chymase.[70-73] Although some evidence supports the possibility that this chymase is expressed in endothelial, vascular or cardiac muscle cells, it is clear that mast cells have far greater capacity to store chymase than any other cell type. Thus, it is probable that most chymase in extracts of human heart muscle, as in other tissues, arises from MC_{TC} mast cells, which are easily detected in heart tissues. History was repeating itself, in the sense that a similar convergence occurred twice in the 1970's in connection with the major mast cell chymotryptic protease of rat connective tissue, mast cell protease I, which was initially thought to be intrinsic to skeletal muscle.[74,75] Similarly, an enzyme extracted from rat intestine, thought to function to degrade intracellular pyridoxal phosphate-dependent enzymes, was subsequently found to be made and stored by an intestinal mast cell sub-population, then called "atypical".[76] These events in the history of mast cell peptidases are worth recounting if only because they remind us that the capacity of mast cells to store peptidases is prodigious; so much so that a minor cell in a tissue like skeletal muscle can produce the lion's share of certain peptidase activities in tissue extracts.

Although human cathepsin G was shown long ago to be capable of selectively hydrolyzing angiotensin I to generate angiotensin II[68] and shown later to be an abundant product of MC_{TC} mast cells,[77,78] rather little is known of its importance in generating angiotensin II in vivo and even less known of its role in other animals. A preliminary comparison of the properties of mouse and human enzymes suggests that the former is more active and narrower in specificity, being more purely chymotryptic. In humans, cathepsin G is a major product of neutrophils (and to a lesser extent, macrophages), in addition to being a product of MC_{TC} mast cells. Thus, it may contribute to the generation of angiotensin II in neutrophilic as well as mast cell-mediated pathologies. In this connection, it may be helpful that potent, dual inhibitors of human chymase and cathepsin G have been generated.[79,80] In humans, chymase can generate angiotensin II while bound to macroglobulin, which prolongs chymase's duration of action and escorts chymase away from site of generation, where it can be detected in blood.[44] Chymase released into interstitial fluid or serum is much more likely to be trapped in this macroglobulin-bound but active form than to be inactivated irreversibly by serpin-class inhibitors, for which chymase has little affinity. Cathepsin G, on the other hand, is susceptible to α_1-antichymotrypsin.

In mice, notwithstanding the apparent redundancy of chymase-like peptidases, it appears that mMCP-4 makes the major contribution, as suggested by studies in mMCP-4-null mice.[81] However, mMCP-4 is not as selective as human chymase for the Phe_8-His_9 bond of angiotensin I and is both an activator and inactivator of angiotensin.[82] Nonetheless, as established by the work of Husain and Dell'Italia and others, mMCP-4 and/or similar chymases appear to be responsible for ACE inhibitor-resistant generation of angiotensin II in mice and for most extravascular generation of angiotensin II in heart muscle,[83] as previously shown in dogs.[84] Although there is potential for production of mMCP-4 and other chymases by nonmast cells, the marked reduction of interstitial angiotensin II generation in mast cell-deficient $Kit^W/Kit^{W\text{-}v}$ mice suggests that most of the extravascular angiotensin II-generating capacity originates from mast cells.[83] Presumably, the existence of this extravascular pathway serves a homeostatic purpose, for example the control of blood pressure. However, many studies in this active area of research focus on potentially deleterious effects such as hypertension (as shown in transgenic mouse expressing rat vascular chymase),[85] restenosis, fibrosis, worsening lung injury in ARDS and cardiac arrhythmias.[83,86-88]

Mast Cell Renin

Studies by Mackins and colleagues[89,90] establish that mast cells can store renin, which is released by mast cell activators like compound 48/80 and allergen. Renin boosts local generation of angiotensin I, which is then processed to angiotensin II by ACE or chymase-like peptidases. Presumably, mast cell renin could serve homeostatic functions, although existing studies focus on potential pathological contributions. In perfused mouse hearts, mast cell deficiency is associated with less "spillover" of renin and fewer malignant ventricular arrhythmias after ischemia-reperfusion. Intriguingly, much of this work on cardiovascular implications of mast cell renin release has been conducted in guinea pigs,[89,90] which appear to lack angiotensin II-generating chymase.[89] Whether mast cell-derived renin is important in myocardial ischemia and arrhythmias in humans needs to be established.

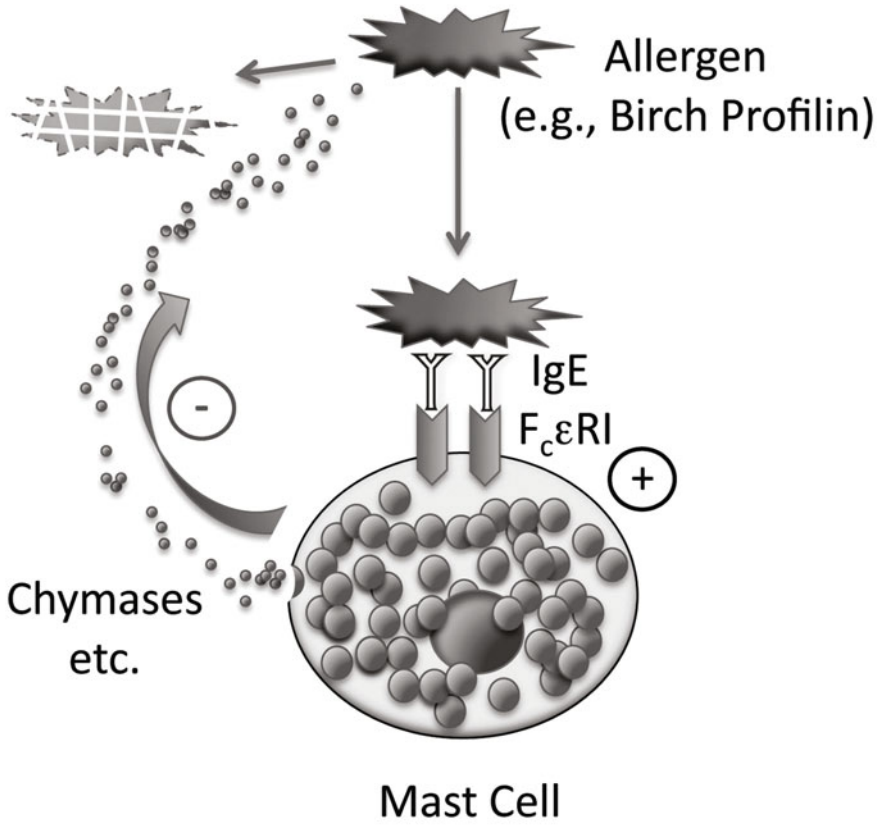

Figure 2. Self-termination of mast cell activation by allergen. Multivalent protein allergens like birch profilin recognized by allergen-specific IgE bound to high-affinity IgE receptor on the surface of mast cells stimulates degranulation and release of epitope-destroying proteases, such as chymases. This is a negative feedback loop that reduces allergic inflammation by destroying allergen.

Mast Cell Proteases, Deworming and Gut Homeostasis

Perhaps the earliest in vivo evidence that a mast cell peptidase serves host defense was provided by Miller and colleagues, who developed a mouse deficient in the mucosal/intraepithelial mast cell chymase mMCP-1.[91] This was the first mammal engineered to lack a mast cell serine peptidase. These *Mcpt1*-null mice have difficulty expelling *Trichinella spiralis*,[91] which is a parasitic roundworm that infects a variety of mammalian hosts and causes trichinellosis; a disease characterized most dramatically in humans by muscle inflammation from tissue deposition of larvae. MMCP-1-expressing mast cells increase dramatically in mice after worm infestation and expulsion of *Trichinella spiralis* accompanies or precedes mMCP-1 release into intestinal mucosa and lumen. This presumably increases intestinal inflammation over the short term but, by expelling worms more quickly, restores gut health sooner. Curiously, mMCP-1's de-worming function does not extend to all nematodes. For example, although mice infected with *Nippostrongylus brasiliensis* develop impressive jejunal mastocytosis and dramatic increases in gut content

of mMCP-1, worm burden is unaffected by lack of mMCP-1 in *Mcpt1*-null mice.[91-94] However, other mast cell proteases may be important. Indeed, recent studies suggest that the related β-chymase, mMCP-4, plays a more general role in regulating gut barrier function, including permeability and epithelial migration. This is indicated by the small intestine phenotype in *Mcpt4*-null mice; and in mast cell-deficient mice with mast cell populations re-established by adoptive transfer of BMCMC originating from wild-type and *Mcpt4* $^{-/-}$ mice.[95] By activating PAR-2, mast cell tryptases may play similar roles in the large intestine.[96]

Chymases and Control of Allergic Airway Inflammation

It is not yet clear whether the overall effect of mast cell chymases released during airway allergen challenge is to promote or oppose allergic inflammation (or both: e.g., pro-inflammatory in the early phase and anti-inflammatory in late or chronic phases). Although it is often assumed that release of chymase will augment inflammation, several experiments suggest mechanisms by which the actions of chymases could reduce or terminate inflammation. For example, chymases could disarm allergens by cleaving them, as occurs with canine chymase degrading birch profilin and destroying epitopes recognized by IgE[97] (see Fig. 2). Furthermore, human mast cell chymase (and cathepsin G) destroy several cytokines associated with perpetuation of allergic inflammation.[41] Indeed, the idea that human chymase opposes inflammation received further support from the observation that chymase-containing mast cells in the outer wall of small airways correlate with better lung function in asthmatics.[98] In a model of asthma, anti-inflammatory activity was attributed to a specific chymase, mMCP4, based on the finding of increased airway responsiveness, inflammation and smooth muscle volume in *Mcpt4* $^{-/-}$ mice.[99] A potential mechanism for increased smooth muscle volume relates to degradation of perimyocyte matrix and reduced proliferative responses to growth factors.[100] Such actions may be related to pro-apoptotic effects of chymases on vascular smooth muscle,[101,102] which may increase susceptibility to aneurysm formation.[103] On the other hand, inhibitors of human chymase and cathepsin G oppose development of airway hyper-responsiveness and early- and late-phase rises in airway resistance in *Ascaris suum*-sensitized sheep challenged with allergen. In mice exposed to tobacco smoke, these inhibitors reduce lung neutrophilia and production of neutrophil chemoattractant.[79,80] Explanations for the observed differences in conclusions drawn from genetic versus pharmacological models likely relate, in part, to differences between mammals in the degree of chymase (and cathepsin G) redundancy along with variations in enzymological properties, inhibitor susceptibilities and cell-selective expression within and between mammals (see ref.4).

Tryptases and Control of Bacterial Infection

This topic has been reviewed recently[104] thus is only briefly summarized and updated here. Among the most persuasive early evidence that mast cells control bacterial infection in vivo came from studies of responses of mast cell-deficient mice to enterobacteria.[105,106] In peritoneal infections, release of TNFα from mast cells early in infection is needed for timely recruitment of neutrophils to contain infection. Subsequent studies revealed that mast cell-derived IL-6 helps to contain peritoneal and lung infections with *Klebsiella pneumoniae* and other gut organisms.[45,107] The possibility that secreted

mast cell proteases could also help to control gut infections was suggested by studies in mice showing that the mouse tryptase, mMCP6, but not the related protease mMCP7, attracts neutrophils when injected into the peritoneal cavity.[108] These studies furthermore established that *Mcpt6* $^{-/-}$ mice clear *Klebsiella pneumoniae* inefficiently and are more likely than *Mcpt6*$^{+/+}$ mice to die following injection of *Klebsiella pneumoniae* into the peritoneal cavity.[109] Other studies provide glimpses of some of the subtleties of mast cell protease contributions by establishing that reduced levels of active mast cell tryptase in *Dppi* $^{-/-}$ mice correlate with increased gut bacterial load following cecal ligation and puncture. This was associated with improved short-term survival; possibly related to higher levels of immunoprotective IL-6, which can be degraded by mMCP-6.[45] Thus, although mouse studies clearly establish that some mast cell tryptases help to control and contain certain types of bacteria, the impact on survival may depend on the nature and virulence of the organism and, in some cases, tryptases may lower survival. In any case, several other mast cell factors, including TNFα and IL-6, are also important. Studies with human mast cell tryptases are more limited, although it has been shown that active human βI tryptase (but not α tryptase) attracts neutrophils when introduced to mouse airways.[110] Whether mast cell tryptases are in fact major weapons in human antibacterial defenses is an unanswered question, the answer to which may determine whether pharmaceutical strategies involving systemic blockade of tryptase activity will block mast cell-mediated inflammation at the expense of creating serious immune deficits. In this regard, the failure to identify humans that are entirely bereft of active mast cell tryptases, despite the high frequency of deficiency alleles in most populations, is consistent with, although not proof of, key roles in host defense.[111-113]

PRO-INFLAMMATORY ROLES OF MAST CELL PEPTIDASES

Tryptases

Allergic Inflammation of Airway and Skin

Tryptases are released with histamine from human skin mast cells in acute and chronic in vivo responses to allergen.[114,115] Levels of immunoreactive tryptases also increase in the airway in asthma[116] and transcripts encoding tryptases are among the most abundant and upregulated gene products in brush biopsies of asthmatic bronchial epithelium, consistent with intraepithelial mast cell migration or local proliferation.[38,117] It is not known if tryptases detected by immunoassays in airway fluids (or in blood, for that matter) are active. Overall, evidence from animal models as well as humans is compelling that tryptases, released during allergic inflammation, are not only markers of mast cell activation, but also contribute to resulting pathology. For example, small molecules designed to inhibit human tryptases markedly reduce airway eosinophilia and goblet cell hyperplasia in a mouse model of asthma.[118] Similar findings are reported with nafamostat,[119] which is a highly potent, although not entirely selective inhibitor of tryptases.[120] Several lines of evidence suggest that tryptase is a bona fide in vivo target of nafamostat. For example, nafamostat reduces scratching in mice induced by skin injection of tryptase, or by the mast cell-degranulator compound 48/80.[17] Nafamostat's effects on scratching are not seen in mast cell-deficient mice and involve PAR-2, since the effects of tryptase and compound 48/80 on scratching are inhibited by PAR-2 antagonists. The simplest explanation of

these findings is that tryptase from mast cells activates PAR-2 on nerves involved in itch pathways. Note that leeches make a potent and selective tryptase inhibitor,[121] which is indirect evidence of a role for tryptase in signaling the presence of the leech, perhaps by activating neural itch and pain pathways or by spreading the degranulation signal.

Published studies of the effect of tryptase inhibition in humans are few. However, a topical (aerosolized) tryptase inhibitor reduced late-phase bronchoconstriction in a small study involving mild human atopic asthmatics[122] and decreased nasal symptoms and eosinophilia in humans with allergic rhinitis.[123] Several pathways by which tryptases may promote asthmatic bronchoconstriction have been proposed. However, it is not yet clear which are the most important. One potential mechanism, proposed early on, is the tryptase-mediated destruction of vasoactive intestinal peptide.[6,25-27] A possibly distinct pathway involves augmentation of bronchoconstriction by histamine and other airway smooth muscle agonists, which is a phenomenon manifest in muscle bath preparations of bronchi from dogs and humans.[19,20,22] Tryptase released from one mast cell under the influence of allergen also may promote degranulation of nearby mast cells, as suggested by mast cell-stabilizing actions of some tryptase inhibitors[124,125] and by the provocation of histamine release in sheep or guinea pig skin or airway by injected or inhaled human tryptase.[23,126-129] The early history of pharmaceutical interest in and development of tryptase inhibitors was thoroughly reviewed by Cairns[130] and will not be rereviewed here. Notwithstanding the pharmaceutical interest in blocking more acute affects of tryptases on inflammation and smooth muscle constriction, the chronic affects on airway remodeling, including growth of airway fibroblasts,[131-134] smooth muscle[135] and vessels,[136] which may be responsible for bronchodilator-resistant airway obstruction, also provide rationales for therapeutic inhibition. The extent to which tryptase activation of PAR-2 is involved in allergic inflammatory, bronchoconstrictor and remodeling responses is not yet clear.

Arthritis and Inflammatory Bowel Disease

A role for mast cell tryptases in arthritis is suggested by reduced inflammation in tryptase-deficient mice in two models of arthritis.[137,138] One of these models (methylated bovine serum albumin/IL-1β-induced) seems to require two tryptase gene products, namely, mMCP-6 and -7, for full expression of the inflammatory phenotype.[137] These recent findings support prior speculation about the importance of mast cell products in arthritis based on studies in mast cell-deficient mice and the finding of tryptase-expressing mast cells in arthritic joints.[139-142] The mechanisms by which mast cell tryptases contribute to various forms of experimental arthritis remain to be established and, of course, tryptases and mast cells are not the sole factors contributing to the phenotype.[141,143] The importance of mast cells and tryptases to the pathogenesis of related human afflictions like rheumatoid arthritis also remains to be clarified. The picture is, in some respects, clearer in regard to inflammatory bowel disease, especially ulcerative colitis, which has been long associated with increases in mast cell numbers and activation in affected tissues.[144,145] At least two pharmacological lines of evidence suggest that tryptases released from mast cells contribute to the pathology of ulcerative colitis: (1) a human trial of a β-tryptase-selective inhibitor, given systemically by subcutaneous injection, appeared to reduce gastrointestinal symptoms in subjects with ulcerative colitis[146] and (2) treatment with the tryptase inhibitor nafamostat decreased the severity of pathological findings in a rat model of colitis caused by trinitrobenzene sulfonic acid.[147]

Chymases and Cathepsin G

Allergic Inflammation in Airway and Skin

The ability of human chymase and cathepsin G to cleave angiotensin I selectively at Phe_8 to generate angiotensin II which, in addition to being a homeostatic process assisting support of blood pressure, can also be pro-inflammatory, was summarized earlier. However, both enzymes have effects that are more classically inflammatory, especially by promoting tissue swelling. For example, dog chymase, injected into dog skin, increases the size of histamine-induced wheals without inducing wheals by itself.[148] Furthermore, inhibition of chymase activity in vivo reduces the size of wheals generated by mast cell-degranulating agents. The mechanisms of these effects are not known, but could include breakdown and untethering of extracellular matrix, so that fluid extravasated under the influence of histamine travels farther than it would do otherwise. Both chymase and cathepsin G are fairly omnivorous and can separate the dermal-epidermal junction by degrading a variety of matrix proteins,[149] as well as by activating MMPs.[49] Possibly, these enzymes also destroy extracellular histaminases so that histamine levels are higher and more sustained. Chymase and cathepsin G also can stimulate gland secretion.[150,151] Indeed, in human airways, chymase-positive mast cells are a high fraction of mast cells lying within 20 μm of submucosal glands.[152] The proteolytic activity of human chymase extends to albumin;[153] however, this cleavage would not increase oncotic pressure because the nicked fragments remain joined by disulfide linkages. Acting subacutely or chronically, chymase-like peptidases also may promote inflammatory angiogenesis, as suggested by sponge granulomas in hamsters[154] and by mast cell-dependent angiogenesis in a model of skin carcinogenesis.[49] The recently reported inhibition of several animal models of allergic and non-allergic inflammation by inhibitors of chymase and cathepsin G[80] further suggests that these enzymes are broadly capable of promoting inflammation. Possibly, they act synergistically when released from human mast cells, where they are usually found in the same granules, because their substrate preferences only partially overlap.

Ischemia-Reperfusion Injury, Aneurysms and Vascular Stenosis

A mouse model of irreversible ischemia-reperfusion injury suggests that a specific mouse mast cell protease, mMCP-5, is responsible for irreversibly injuring skeletal muscle.[155] This work establishes the principle that a mast cell protease can be cytotoxic in the context of ischemic inflammation, which is associated with mast cell activation. The mechanism by which muscle is damaged by mMCP-5, which is an elastolytic peptidase with no known functional equivalent, in human mast cells is not clear. However, the potential for chymase-related mast cell peptidases to damage and alter vessels themselves has gathered increased experimental support over the past few years. After initial studies suggested a role for mast cells in promoting arterial enlargement in a neutrophil elastase-induced mouse model of aortic aneurysm,[156] subsequent studies showed at least partial dependence on mast cell expression of mMCP-4.[103] The mechanism by which this chymase promotes aneurysm formation in this model is hypothesized to include inflammatory activation of vessel wall-weakening MMPs and cathepsins and direct stimulation of aortic smooth muscle cell apoptosis. On the other hand, there is strong in vivo pharmacological evidence from studies in a variety of models of vascular injury that chymase-like enzymes promote cardiovascular remodeling, fibrosis and stenosis in response to injury.[62,88,157-160]

Dipeptidyl Peptidase I and Other Thiol Cathepsins

Membrane-bound mast cell secretory granules, harboring biogenic amines, proteases and proteoglycans, are related to lysosomes and thus partly may serve lysosomal functions. Indeed they contain some lysosome-associated proteases. Cathepsin G is not a classic cathepsin because it is a serine, not thiol, peptidase with expression restricted to specialized granules of mast cells and myelomonocytic cells, especially neutrophils. Another granule peptidase, dipeptidyl peptidase I (DPPI; cathepsin C) also is atypical. Although it is expressed in many cells, it is much more abundant in mast cells, myelomonocytes and other specialized granulated cells, like cytotoxic T and natural killer cells. In uninflamed dog airway, mast cells are the dominant cell type staining positively for DPPI.[161] Like other proteins of secretory granules, DPPI can be secreted[162] and it may cleave extracellular targets.[161] However, it is not highly destructive because its activities are restricted compared to other thiol cathepsins by an "exclusion domain", ensuring preference for cleaving N-terminal dipeptides.[163] These attributes suggest that DPPI is likely to serve an intragranular function not related to general protein degradation or typical lysosomal activity. At present, DPPI's major identified role is to activate granule-associated immune cell peptidases related to chymases, cathepsin G, lymphocyte granzymes and neutrophil elastase. It accomplishes this by removing the N-terminal pro-dipeptide that is a shared attribute of these enzymes.[164-167] Given the effects of genetic inactivation of DPPI on activation of an impressive range of conserved immune serine peptidases, the phenotype might be expected to be more severe than it is. In fact, genetic deletion or inactivation of DPPI is not lethal to mice protected from infections, but DPPI-deficient mice have a variety of immune deficits and altered responses to sepsis,[14,168] including improved short term survival following cecal ligation and puncture.[45] Humans with defects in DPPI have chronic periodontal infections.[169] Mice lacking DPPI have little if any mast cell chymase activity, although at least one chymase (mMCP-4) is present in mast cell granules as an inactive pro-enzyme.[166] The effect on mouse tryptase mMCP-6 is less dramatic, with activity being reduced but present.[166] This is perhaps not surprising, given that tryptases possess a much longer pro-peptide than chymases, granzymes and neutrophil elastase-like hydrolases.[170,171] Schwartz and colleagues suggest that the pro-peptide is removed from human β-tryptases by tandem cleavages initiated by autocatalysis to generate a remnant pro-dipeptide removed by DPPI.[172] The extent to which human chymases and tryptases depend on DPPI for activation in vivo remains to be determined. Mast cells do express classical lysosomal thiol cathepsins, including cathepsin S, which can influence levels of chymase and/or carboxypeptidase independently of DPPI in mouse mast cells.[173,174] Thus, although DPPI has received more attention in studies of mast cell biology to date, other thiol cathepsins may be important to mast cell function.

CONCLUSION

Mast cell peptidases and proteases, particularly those that are stored in secretory granules and released by exocytosis, can augment or suppress inflammation, depending on the tissue and context in which they are released. Mounting evidence also suggests that they play critical, supporting roles in protective, innate immune responses by inactivating toxic endogenous peptides associated with inflammation and sepsis, disarming peptides in venoms and supporting blood pressure via generation of angiotensin II. These

contributions and the pathways leading to them remain active areas of basic and clinical investigation. In summary, proteases—which are the major proteins stored and released by mast cells—are immunoregulatory proteins that contribute in a variety of important ways to inflammation, protective host responses and overall tissue homeostasis.

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CHAPTER 13

MAST CELLS IN LUNG INFLAMMATION

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Abstract: Mast cells play an important role in the lung in both health and disease. Their primary role is to initiate an appropriate program of inflammation and repair in response to tissue damage initiated by a variety of diverse stimuli. They are important for host immunity against bacterial infection and potentially in the host immune response to non small cell lung cancer. In situations of ongoing tissue damage, the sustained release of numerous pro-inflammatory mediators, proteases and cytokines, contributes to the pathophysiology of lung diseases such as asthma and interstitial lung disease. A key goal is the development of treatments which attenuate adverse mast cell function when administered chronically to humans in vivo. Such therapies may offer a novel approach to the treatment of many life-threatening diseases.

INTRODUCTION

In this chapter, we will examine the role of mast cells (MCs) in the initiation and maintenance of airway and parenchymal inflammation and fibrosis. MCs play an important role in host defense against pathogens, particularly bacterial infection and in tissue repair through the synthesis and release of numerous pro-inflammatory mediators, proteases and cytokines, the pattern of which varies depending on the stimulus. In healthy human lung, MCs are ubiquitous in the airways and parenchyma, but redistribute to key tissue structures in disease. Their location at the interface between the external environment and lung tissue places them in the ideal location to respond rapidly to perceived tissue insults. MCs can respond to many diverse stimuli (Fig. 1) and it is our view that their primary role is to initiate an appropriate program of inflammation and repair in response to tissue damage, but that if this stimulus becomes chronic, than tissue dysfunction and fibrosis ensue. Human lung mast cells (HLMCs) achieve their biological effects through cell-cell interactions and the release

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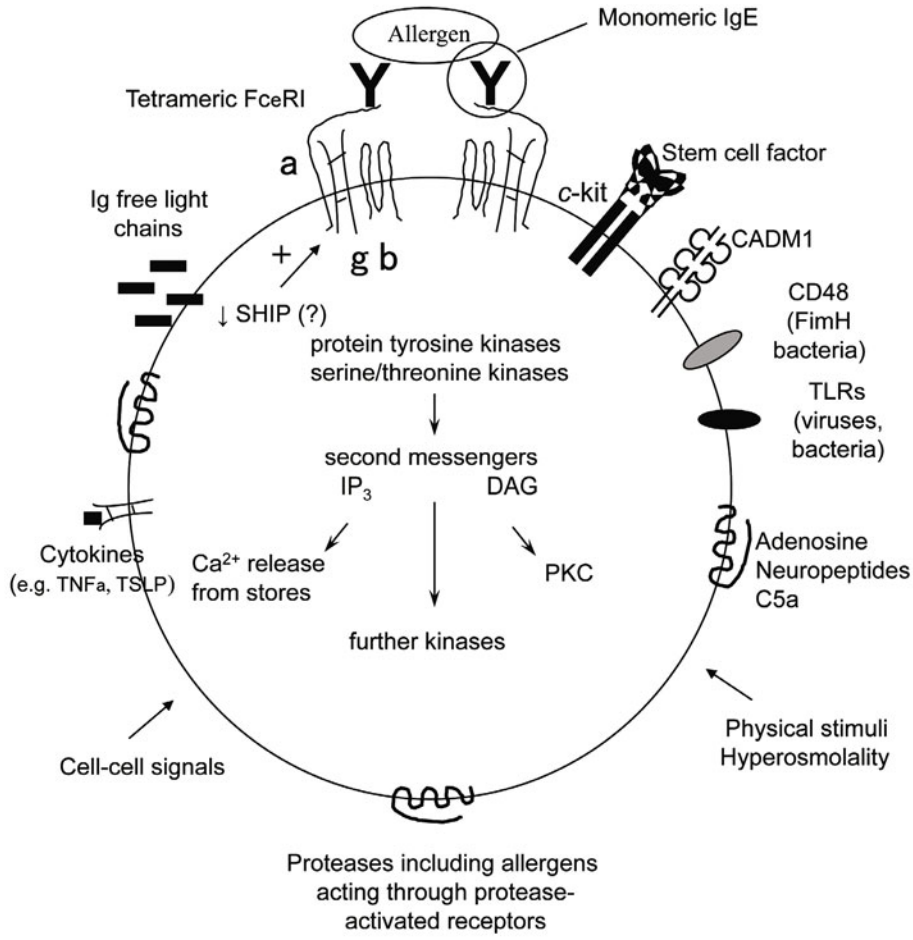

Figure 1. Potential mechanisms of human lung mast cell activation.

of numerous autacoid mediators (histamine, prostaglandin [PG]D_2, leukotriene [LT]C_4), proteases and multifunctional cytokines and chemokines. The biological activities of these mediators are summarized in Tables 1-3. The release of these mediators is not all-or-none, but highly regulated and stimulus-specific. This allows the MCs to interact with structural cells, as well as cells of both the innate and acquired immune systems.

MAST CELL RECRUITMENT TO THE LUNG

The mechanisms of MC homing to human lung are poorly understood and the primary signals are unknown. However, MC expansion in sensitized murine lung is induced by Type 2 NKT cells and IL-9 after allergen challenge and PGE_2, produced by nasal mucosa.[1,2] In addition, systemically available sphingosine 1-phosphate (S1P) increases

Table 1. The classical human mast cell mediators and their biological effects

Mediator	Activity
Histamine (stored)	Bronchoconstriction; tissue edema; ↑ vascular permeability; ↑ mucus secretion; ↑ fibroblast proliferation; ↑ collagen synthesis; ↑ endothelial cell proliferation, dendritic cell activation
Heparin (stored)	Anticoagulant; storage matrix for mediators; sequestering of growth factors from degradation; fibroblast activation; endothelial cell migration
Tryptase (stored)	Degrades respiratory allergens and cross-linked IgE; generates C3a and bradykinin; degrades neuropeptides; increases BHR and ASM contractility; indirectly activates collagenase; ↑ fibroblast proliferation and collagen synthesis; epithelial activation, potentiates mast cell histamine release; neutrophil recruitment; TGF-beta activation
Chymase (stored)	↑ mucus secretion; ECM degradation, Type I procollagen processing, angiotensin II synthesis, ↓ T-cell adhesion to airway smooth muscle, activates IL-1b, degrades IL-4, releases membrane-bound SCF
PGD_2 (synthesized)	Bronchoconstriction; tissue edema; ↑ mucus secretion; dendritic cell activation; chemotaxis of eosinophils, Th2 T cells and basophils via the CRTH2 receptor
LTC_4/D_4 (synthesized)	Bronchoconstriction; tissue edema; ↑ mucus secretion; enhances IL-13-dependent airway smooth muscle proliferation; dendritic cell maturation and recruitment; eosinophil IL-4 secretion, mast cell homing, mast cell IL-5, IL-8 and TNFα secretion; tissue fibrosis

MC number in bronchoalveolar lavage (BAL) fluid in mice.[3] Mediators from mature MCs may also recruit further immature MC progenitors[4] (see Tables 1 and 2). In addition, the lung MC population may expand in pathological conditions such as fibrosis via several stimuli, including stem cell factor (SCF), S1P and other growth factors. There is evidence that several factors may lead to the redistribution of MCs in human lung. For example, HLMCs express CXCR3, particularly on the cells within the ASM bundles in asthma, which in turn preferentially express the chemotactic ligand CXCL10.[5]

MAST CELLS IN HEALTHY LUNG

HLMCs show a marked heterogeneity within the tissue with variability in their size and shape, protease and cytokine content and response to various stimuli (reviewed in ref. 6). In comparison with MCs from other sources, HLMCs display distinct differences in phenotype distribution, biomarker expression patterns, chemokine receptor profiles and released mediators.[7-12]

HLMCs belong to either a predominant tryptase+ (MC_T) or tryptase/chymase+ (MC_{TC}) subtype. The rare chymase-only (MC_C) subtype has also be identified within the lung in several studies.[7,9,13] Isolated lung MC_T are smaller and produce lower amounts of the autacoid mediators histamine and PGD_2 compared with MC_{TC} cells.[10,14,15] In situ,

Table 2. Biological activity of human mast cell-derived chemokines in vitro

Chemokine	Target Cells	Biological Effects
CCL1	T cells	T-cell recruitment
CCL2	Mast cells	Chemotaxis
	Epithelial cells	Proliferation, chemotaxis
	Fibrocytes	Chemotaxis
	T cells	→Th2 phenotype
	Eosinophils	Chemotaxis
	Monocytes	Chemotaxis
	Basophils	Activation, mediator release
CCL3	Mast cells	Activation, mediator release
	T cells	→Th1 phenotype and chemotaxis (Th1-selective)
	Macrophages	Differentiation
	Neutrophils	Chemotaxis (in vivo) and
	Eosinophils	cytotoxicity
	Monocytes	Chemotaxis
	Basophils	Chemotaxis
		Activation, mediator release
CCL4	T cells	→Th1 phenotype and chemotaxis (Th1-selective)
	Eosinophils	Chemotaxis
	Neutrophils	Chemotaxis (in vivo)
CCL5	Mast cells	Chemotaxis?
	T cells	→Th1 phenotype and chemotaxis (Th1-selective)
	Eosinophils	Chemotaxis
	Monocytes	Chemotaxis
CCL7	Eosinophils	Chemotaxis
	Monocytes	Chemotaxis
	Basophils	Activation, mediator release
CCL12	Fibrocytes	Chemotaxis
	Monocytes	Chemotaxis
	Eosinophils	Chemotaxis
	Lymphocytes	Chemotaxis
CCL17	T cells	Chemotaxis (Th2-selective)
CCL19	Airway smooth muscle	Chemotaxis
CCL20	Dendritic cells	Chemotaxis
	T cells	Chemotaxis
CCL22	T cells	Chemotaxis (Th2-selective)
CXCL5	Neutrophils	Chemotaxis
CXCL8	Mast cells	↓ chemotaxis, ↓ mediator release
	Endothelial cells	Proliferation, survival, chemotaxis, angiogenesis
	Neutrophils	Chemotaxis
	Eosinophils	Chemotaxis after priming with IL-3, IL-5 or GM-CSF

Table 3. Biological activity of human mast cell-derived cytokines in vitro

Cytokine	Target Cells	Biological Effects
IL-4	B cells	proliferation; ↑ MHC class II, ↑ CD40, ↑ CD25; ↑ IgE, ↑ IL-6
	T cells	Proliferation, →Th2 cell phenotype
	Monocyte/macrophages	↓ H_2O_2 and O_2^-, ↓ parasite killing, ↓ tumoricidal activity; monocyte → macrophage differentiation; ↓ IL-1, IL-6, IL-8 and TNF ↑ MHC class II and CD23; 15-lipoxygenase expression
	Eosinophils	Transendothelial migration
	Fibroblasts	Proliferation, chemotaxis, ↑ ECM; ↑ ICAM-1
	Endothelial cells	Proliferation, ↑ VCAM-1, ↓ ICAM-1
	Mast cells	↑ FcεRI, ↑ ICAM-1
IL-3, IL-5, GM-CSF	Eosinophils	Growth, adhesion, transendothelial migration, chemotaxis, activation and prolonged survival
IL-6	B cells	Ig secretion including ↑ IgE
	T cells	Growth, differentiation
	Mast cells	Survival
IL-13	B cells	IgE synthesis
	Monocyte/macrophages	As for IL-4
	Eosinophils	Activation, survival
	Endothelial cells	↑ VCAM-1
TNFα	Monocyte/macrophages	↑ cytotoxicity, chemotaxis, prolonged survival
	T cells	Class II antigen and IL-2R expression, proliferation ↑ cytotoxicity, chemotaxis
	Neutrophils	↑ cytotoxicity, oxidant production
	Eosinophils	↑ E-selectin, ↑ ICAM-1, ↑ VCAM-1;
	Endothelial cells	↑ intravasation of most leucocytes
	Fibroblasts	Growth, chemotaxis, ↓ collagen, ↑ collagenase, ↑ IL-6, ↑ IL-8
	Mast cells	Histamine and tryptase secretion
SCF	Mast cells	Growth, differentiation, survival, chemotaxis
NGF	B cells	Differentiation, proliferation, ↑ Ig
	T cells	Differentiation, proliferation
	Eosinophils	Proliferation
	Basophils	Activation, mediator release
	Neutrophils	Chemotaxis, survival, mediator release
	Monocyte/macrophages	Proliferation, mediator release
	Fibroblasts	Migration, contraction
	Smooth muscle cells	Migration, contraction, proliferation

continued on next page

Table 3. Continued

Cytokine	Target Cells	Biological Effects
	Mast cells	Differentiation, survival, activation, mediator release
TGFβ	Smooth muscle cells	Differentiation, activation
	Epithelial cells	Inhibition of proliferation
	Endothelial cells	↑ angiogenesis
FGF-2	Fibroblasts	Proliferation
	Endothelial cells	↑ angiogenesis
IFNα	NK cells	↑ cytotoxicity
	Macrophages	Development, maturation
	Dendritic cells	Activation, maturation, ↑ IFNγ
	T cells	↑ survival of activated T cells, ↑ Th1 phenotype
TSLP	Dendritic cells	induction of Th2 immunity
	Mast cells	↑ IL-13 expression

the size of lung MC_T and MC_{TC} cells varies according to the location.[16] In addition, MC_{TC} cells accumulate other peptidases, express CD88 and a distinct set of cytokines and chemokine receptors.[8-10,17,18] Their properties are summarized in Table 4. The MC_T phenotype is typically found in the lung parenchyma, bronchial epithelium and the bronchial lamina propria, whereas the MC_{TC} phenotype localizes around blood vessel walls.[8,9,16,19] Andersson et al identified several site-specific populations within conventional MC_T and MC_{TC} types on the basis of expression patterns for the IgE receptor, IL-9 receptor, renin, histidine decarboxylase, VEGF, basic FGF (bFGF, FGF2), 5-lipoxygenase and leukotriene C_4 synthase. Interestingly, renin expression is observed predominantly in the MC_T subtype and only in pulmonary vascular MC_{TC} cells. The relatively high expression of the high-affinity IgE receptor and histidine decarboxylase, present in central airway MC_T and MC_{TC} cells, with IgE receptor virtually absent in alveolar MCs, are likely to be of particular physiological importance.

A differential expression pattern of proteases among MCs and the varying anatomical location of these cells indicate that they will have distinct roles in normal lung physiology and disease, but these remain poorly understood. MCs subtypes develop in the presence of specific factors and show a high degree of plasticity—a rapid conversion of cultured MC_{TC} cells to MC_T has been reported in coculture with human airway epithelium.[20] Hence, it is perhaps not surprising that MCs adopt the MC_T subtype in close proximity to epithelial cells in the alveoli and bronchi. In contrast, coculture of MC_T cells with endothelial cells transforms them into the MC_{TC} subtype,[21] which may explain the predominance of the MC_{TC} subtype around blood vessels.

Taken together, the existence of site-specific subpopulations of HLMCs is likely to be a response to local environmental cues affecting gene expression, resulting in appropriate physiological function.[16] For example, renin expression in combination with chymase, converting AngI into AngII, in MC_{TC} cells close to pulmonary vasculature, as well as VEGF expression in MCs around pulmonary vessels, suggests involvement of these MCs in local regulation of vascular function.

Table 4. Phenotypes and localization of lung mast cells in normal lung and in disease

Mast Cells	Total Population	MCT Subtype	MCTC Subtype
Parameter		Normal Lung	
Location and percentage	MC_T, MC_{TC} and rare MC_C.[7] MC density is increased in small airways and alveolar parenchyma compared with central airways.[16]	85-95% in the alveolar walls, bronchioles and bronchi.[8,9,16,19]	4-15% in alveolar attachments, small airway outer wall and in bronchi,[8,9,16,19] ~50% around pulmonary vessels.[16]
Protease content	Decreased renin in central airways compared to other locations.[16]	Tryptase.[8]	cathepsin G,[17] carboxypeptidase A.[8]
Specific biomarkers	Increased FcepsilonRI and histidine decarboxylase, decreased 5-lipoxygenase in central airways compared to other locations.[16]		CD88,[10] increased leukotriene C4 synthase in small airways and around blood vessels.[16]
Cytokines, chemokines and receptors	CCR3 and CXCR1 in ~15% of MCs; CXCR3 and CXCR4 in ~30% MCs.11 Increased IL9R in small airways and around pulmonary vessels compared to central airways; increased VEGF around pulmonary vessels.[16]	IL-4(low), IL-5, Il-6, IL-13 (low).[9]	IL-4(high), IL-13 (high),[9] CCR3.[18]
Responsiveness to stimuli		FcepsilonRI, substance P.[10]	FcepsilonRI, C5a, compound 48/80, substance P.[10]

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Table 4. Continued

Mast Cells	Total Population	MCT Subtype	MCTC Subtype
		Asthma	
Location	Increased density and degranulation in epithelium,[87,88,91,93] in lamina propria,[93] the airway mucous glands,[96,97] ASM.[96-98] Increased percentage in small airway outer and inner wall regions, alveolar attachments, peripheral alveolar tissue and large airway in severe asthma.[13] Increased numbers around small blood vessels in bronchi.[99] Increased density in the adventitial layer of small pulmonary arteries in fatal asthma.[100]		Predominant subtype infiltrating ASM (83%).[98] Higher numbers and the MCTC/MCT ratio in alveolar attachments in severe asthma.[13] Increased density around blood vessels in the lamina propria.[99] Increased density in the adventitial layer of small pulmonary arteries in fatal asthma.[100]
Cytokines, chemokines and receptors	7-fold increased TNF-alpha expression.[88] 55% of MCs within the ASM express IL-4 and 17% express IL-13.[101] Increased MC number correlates with CCR1 and CCR4-expression in asthma; CCR1 and CCR4 in 24% and 19% of MCs, respectively.[102] CXCR3 is expressed in 100% of MCs in ASM and 47% of MCs in the submucosa.[5]		100% IL-4-positive vs 60% in control.[9]
Specific biomarkers	Increased amphiregulin[103] and thymic stromal lymphopoietin.[104]		

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Table 4. Continued

Mast Cells	Total Population	MCT Subtype	MCTC Subtype
Functional correlations	MC localization to the bronchial ASM is associated with airway hyperresponsiveness in asthma.[105] The numbers of total MCs and degranulated MCs correlate with the lumen area occupied by mucus.[106]		There was a significant inverse correlation between the number infiltrating the bronchial ASM and the PC 20 for methacholine in the subjects with asthma.[98] The MC_{TC} number and the MC_{TC}/MC_T ratio in the small airway outer wall and alveolar attachments positively correlate with lung function in severe asthma.[13] The density in the lamina propria is related to the vascular area and to the number of $VEGF^+$ MC in mild to moderate asthma,[99] with the MC_{TC}/MC ratio correlated with the PD20 methacholine values.[99]
		COPD	
Location	Reduced net MC density with increased luminal MC number.[107] Reduced density in submucosa and ASM bundle, but increased in glands (did not reach statistical significance).[108]	Lower density in the subepithelial area of central airways vs. controls.[109] Lower numbers in the subepithelial area of airways vs controls.[107]	Higher numbers in both subepithelial area and ASM area of peripheral airways, but not in controls.[109] Increased density in walls of small airways and in alveolar parenchyma vs control;[107] several-fold increase in small airways, small airway epithelium, pulmonary vessels and alveolar parenchyma in very severe COPD.[107]
Specific biomarkers		Increased expression of CD88.[107]	Increased expression of CD88 and TGF-beta and decreased rennin.[107]

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Table 4. Continued

Mast Cells	Total Population	MCT Subtype	MCTC Subtype
		COPD (continued)	
Functional correlations		Cell numbers in the epithelium and subepithelial area of peripheral airways positively correlate with FEV1/VC lung function, the cell percentage in the total airway wall area of peripheral airways positively correlate with higher values of FEV1 and FEV1/VC.[109]	Cell numbers in ASM of peripheral airways positively correlate with FEV1% pred lung function. Higher numbers in the adventitia of peripheral airways tend to correlate positively with FEV1% pred.[109]
		Fibrotic Lung Disorders	
Location	Increased MC number in the alveolar septa and within alveoli.[110] Increased MC density and activation in bronchi.[111-113]		Increased MC_{TC} counts in ASM cells.[113]
Specific biomarkers	increased bFGF in lung interstitium, surrounded by collagen, elastic fibers and ASM/myofibroblast cells.[114-116]		
Functional correlations	MC counts significantly correlate with the degree of fibrosis.[110]		MC_{TC} numbers correlate with accumulation of ASM cells and myofibroblast proliferation.[113]

MAST CELLS IN LUNG DEFENSE

Since the role of MCs in the immune response is addressed in other chapters, here we present only those data which are directly related to respiratory infection and lung inflammation.

Defense against Bacterial Infections

Experimental animal models suggest that MCs are indispensible for the clearing of pulmonary infections caused by respiratory pathogens such as *Klebsiella pneumoniae, Mycoplasma pneumoniae*, *Streptococcus pneumoniae and Mycobacterium tuberculosis*.

Direct contact of MCs with clinical isolates of *K. pneumoniae Clll* decreased bacterial viability after 60 min in vitro, indicating involvement of MCs in the innate immune response.[22] In an acute lung infection model, mast-deficient $Kit^{W/Wv}$ mice had a much higher burden of *K. pneumoniae KP1415* in the lungs compared with wild type or $Kit^{W/Wv}$ mice reconstituted with MCs.[23] Similarly, MC-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice infected with *K. pneumoniae* intranasally have 4-fold worse survival after 4 days compared to wild-type mice.[24] $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice are more likely to die from *M. pneumoniae* compared with wild-type mice.[25] The mycoplasma burden is greater in MC-deficient mice with a 162-fold difference 4 days following infection when compared to wild-type mice. At later time points, bacterial burden is similar in both mouse strains, but neutrophilic inflammation remains severe in MC-deficient mice.[25] This latter observation raises the possibility that MCs may also have an anti-inflammatory role in the context of resolving bacterial infection.

MCs have the ability to bind and engulf bacteria and they also release a number of inflammatory mediators including TNFα, IL-4, IL-6, IL-10 and leukotrienes in response to bacterial infection. Exposure of the rat MC line RBL-2H3 to *M. pneumoniae* induces production of mRNA for IL-4, IL-6 and TNFα within 2 hours.[26] Interestingly, expression of the FcεRI alpha chain expression is required for activation of MCs for the IL-4 production induced by *M. pneumoniae.*[27]

Several MC mediators are crucial for a full antibacterial response against acute infection. TNFα released locally by MCs plays a central role by recruiting neutrophils to sites of infection. TNFα is induced in response to bacterial FimH binding to the CD48 receptor in MCs and contributes to host defense against *K. pneumoniae.*[23] Inhibition of TNFα activity within the lung results in markedly decreased both short- and long-term survival of mice challenged with *K. pneumoniae* intratracheally.[28] Likewise, susceptibility to pneumonia in some strains of mice, infected with *S. pneumoniae*, correlates with the number of MCs and the speed of TNFα release in the lung, which is required for recruitment of circulating leukocytes.[29] MC-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice selectively reconstituted with IL-$6^{-/-}$ or IL-$6^{+/+}$ bone marrow MCs show that IL-6 produced specifically by MCs regulates survival from *K. pneumoniae* infection (peritonitis) by activating neutrophils and enhancing killing of intracellular bacteria by neutrophils.[24] Recently a new protective role of IL-4 produced by MCs has been identified in an acute infection model with life-threatening *Francisella tularensis*.[30] In this model, MCs were the first to infiltrate the lungs of infected animals prior to macrophages, dendritic cells or neutrophils. MCs significantly inhibit bacterial uptake and growth within macrophages specifically via production of IL-4 in response to infection.[30] MC transfer results in a remarkable level of survival of infected $Kit^{W/Wv}$ mice.[30]

Further important MC secreted factors required for host defense are the MC-specific proteases. For example, mice lacking protease-6, similar to human tryptase beta 1, cannot clear *K. pneumoniae* efficiently after peritoneal inoculation.[31] These mice have 8-fold lower survival after 3 days compared with that of wild-type mice. Increased lethality in these mice is associated with higher bacterial burden and lower neutrophil recruitment despite normal MC production of IL-6 and TNFα. Likewise, when MC-deficient $Kit^{W/W(v)}$ mice were given enzymatically active human tryptase beta I or its inactive zymogen before pulmonary infection with *K. pneumoniae*, tryptase beta I-treated mice had ~5 fold fewer viable bacteria in their lungs 6 hours after inoculation relative to zymogen-treated mice.[32] When enzymatically active tryptase beta I, but not tryptase alpha, was instilled into the lungs of mice, neutrophil numbers in BAL increased 100-fold, but methacholine-induced bronchoconstriction was not altered. These data suggest that mMCP-6/tryptase beta I is the primary preformed granule mediator that protects mice from acute bacterial infections. The mechanism of neutrophil recruitment by mMCP-6/tryptase beta I is not clear, but the experimental data suggest that it is not due to IL-8, IL-6 or TNFα,[31,32] but may involve neutrophil chemotactic factors, produced by other cell types stimulated by tryptase.[33]

In summary, experimental models of acute respiratory bacterial infection show that MCs are critical in the early stages of infection and have a profound effect on survival for days after the onset of infection. Mouse models show that MCs contribute to innate defenses against respiratory pathogens.

Defense against Viral Infection

Respiratory viral infections cause accumulation of MCs, as well as T and B lymphocytes during the first days of a symptomatic naturally acquired infection in the nasal mucosa.[34] Exposure of human MCs to respiratory viruses, such as influenza virus (PR8), Type 1 reovirus and respiratory syncytial virus (RSV), induced substantial production of IFNα; a similar response was observed upon stimulation of these cells with the TLR3 ligand, poly I:C.[35] Poly I:C-induced IFNα production involved activation of NF-kappaB, p38 and C-Jun NH2-terminal kinase and mitogen-activated protein kinase. Activation of TLR3 with poly I:C did not induce TNFα, IL-1β, IL-5, or GM-CSF production, in contrast to activation of TLR-2, 4, 5 and 9 by peptidoglycan, LPS, flagellin or CpG, respectively.[35] In addition, treatment with dsRNA alone or with IgE did not lead to MC degranulation.[35] Similarly, degranulation does not occur by direct RSV infection into MCs, however, it was detected in MCs sensitized with specific IgE.[36,37] Furthermore, coculture with RSV-infected lung cancer A549 cells with the MC line HMC-1 induced degranulation in HMC-1 cells which coincided with upregulation of TNFα secretion. In this case, degranulation is likely to be mediated by defensins released from infected epithelial cells.[38] These data suggest that MC degranulation may occur during respiratory viral infection in vivo. In keeping with this, RSV infection increases the release of leukotrienes from MCs in rat airways.[39]

Infections with some viruses, such as RSV or influenza virus, induce virus-specific IgE in humans and experimental models.[37,40,41] IgE, specific to respiratory viruses, is implicated in MC degranulation, resulting in asthmatic and allergic responses in mouse models of allergic diseases.[41,42] Similar to viral infections, respiratory bacterial infections with *Chlamydia pneumoniae*, *Haemophilus influenzae*, *S. pneumoniae* and *M. pneumoniae* often lead to production of specific IgE in a large population of patients.[43-45] Moreover, the prevalence of bacterial-specific IgE is common in both asthma and chronic bronchitis.[44,45] It is therefore plausible that on reinfection, a simultaneous engagement of TLRs, other

bacterial-specific receptors and FcεRI causes a synergistic activation of MCs, further amplified by pro-inflammatory signaling from surrounding cells in lung tissue. This amplified MC response is likely to be involved in asthma exacerbations commonly occurring during viral and bacterial infections and leading to deterioration of airway physiology.

Mast Cell Activation by Pathogens—Mast Cell Toll-Like Receptors

The functional response of HLMCs to bacterial, viral and other ligands has not been studied in detail. HLMCs show distinctive TLR expression profiles compared with MCs from umbilical cord, peripheral blood or skin.[35,46,47] They express relatively high levels of TLR-2, -3, -4 and -7, lower levels of TLR-1, -5 and -10, whereas expression of TLR-6, -8, or -9 is not observed in these cells.[47] In contrast to HLMCs, murine peribronchial MCs strongly express TLR-9.[48]

Exposure of HLMCs to the TLR2 ligand, lipoteichoic acid, reduced both FcεRI expression and IgE-induced degranulation, but the TLR2/TLR6 ligand, peptidoglycan, did not have similar effects.[49] These data suggest that some bacterial TLR2 ligands may modify some aspects of allergic disease. In murine lung MCs, a synthetic ligand for TLR2/TLR1 heterodimers, tripalmitoyl Cys-Ser-(Lys)4, induced JE (murine equivalent to human chemokine CCL2) production via NF-kappaB activation, but did not induce Ca^{2+} mobilization or degranulation.[50] These responses observed in murine cells were in contrast to efficient Ca^{2+} mobilization and degranulation in the human LAD-2 MC line, stimulated with the same TLR2 agonist.[50] $TLR2^{-/-}$ mice displayed increased bacterial burden upon infection with *M. tuberculosis* compared with wild-type mice, whereas reconstruction of the mice with $TLR2^{+/+}$ MCs reduced the bacterial burden, indicating direct involvement of MC TLR2 in the control of lung infection.[51] Adoptive transfer of $TLR2^{+/+}$ MCs to $TLR2^{-/-}$ mice resulted in regulation of the pulmonary levels of IL-1β, IL-6, TNFα, an enhanced Th1 response and activated $CD8(^{+})$ T cell homing to the lungs.[51]

MAST CELLS IN CHRONIC LUNG DISEASE

Asthma

Asthma is an airway disease that is characterized by airway obstruction due to bronchoconstriction, mucus hypersecretion, airway inflammation and edema and remodeling of various airway elements. Chronic MC activation and the relocation of these cells within specific airway structures play a key role in asthma pathogenesis. Increased MCs activation contributes to both chronic inflammation and tissue remodeling (reviewed in ref. 52).

Mast Cells in the Pathophysiology of Asthma

There is compelling evidence, based largely on studies from humans, that MCs play a pivotal role to the pathogenesis of asthma. They secrete a host of pro-inflammatory mediators which are directly relevant to the pathophysiology of asthma (see Tables 1-3). For example, the autacoid mediators, histamine, PGD_2 and LTC_4, induce bronchoconstriction, mucus secretion and mucosal edema and thus contribute to the symptoms of asthma. MCs also synthesize and secrete a number of proinflammatory cytokines (including IL-4,

IL-5 and IL-13), which regulate both IgE synthesis and the development of eosinophilic inflammation; and several profibrogenic cytokines including TGFβ and bFGF. Moreover, the serine proteases tryptase and chymase, which are major secretory products of human MCs, can interact with various cell types and profoundly alter their behavior.

Mast Cells and Human Experimental Allergen-Induced Asthma

Approximately 90% of subjects with asthma under the age of 30 are atopic, with positive skin prick tests to common aeroallergens such as the house dust mite (reviewed in see ref. 53). The role allergen exposure plays in day-to-day chronic asthma is probably over-stated, but the ability of pollen to exacerbate asthma,[54,55] and the ability of anti-IgE therapy to attenuate the disease in some patients indicates that allergy remains a relevant factor in asthma pathophysiology. Bearing this in mind, the technique of acute bronchial challenge with a relatively large dose of allergen in the laboratory has provided a useful model for studying asthma pathophysiology. Following bronchial allergen challenge, most atopic asthmatics and many atopic nonasthmatics experience a rapid fall in pulmonary function (e.g., forced expiratory volume in one second [FEV_1]) which peaks at 10-20 minutes and then gradually recovers over the following 2 hours. This is defined as the early asthmatic response (EAR). In about 50% of subjects, after 4-6 hours there is a further fall in FEV_1, termed the late asthmatic response (LAR). This may last up to 12 hours and in some individuals may be followed by recurring airway obstruction for several days or even weeks (reviewed in see ref. 53).

The EAR: Several vasoactive and spasmogenic mediators are released during the EAR, most of which originate from MCs resident in the airway mucosa. The relative rate of mediator release from HLMCS in vitro is histamine>PGD_2>LTC_4 with one-half maximal release occurring at 2, 5 and 10 minutes respectively.[56] This is reflected in vivo by the recovery of these mediators in BAL fluid within 5-10 minutes following local bronchial allergen challenge.[57-63] It has been calculated that the concentrations of mediators generated are similar to those required to produce bronchoconstriction in vitro[62] or when delivered by nebuliser.[60] Evidence for their role is cemented by studies using potent and selective receptor antagonists, which demonstrate that the EAR is significantly attenuated by antagonists of histamine, LTC_4 and to a lesser extent PGD_2.[64-68]

The MC as a source of these mediators is supported by four lines of evidence. Firstly, the kinetics of IgE-dependent mediator release in vivo parallels that of purified MCs in vitro. Secondly, the presence of MC activation during the EAR is confirmed by the rapid increase in concentrations of the preformed MC-specific protease, tryptase, recovered by BAL within minutes following local bronchial allergen challenge.[58,63] Thirdly, β-agonists such as salbutamol, known inhibitors of MC degranulation when applied acutely in vitro, completely abolish the early reaction and the associated increase in plasma histamine levels.[69-71] Lastly, the EAR is markedly attenuated after pretreatment with anti-IgE,[72,73] confirming that IgE-dependent signaling is critical.

The LAR: In contrast to the acute mediator-induced bronchoconstriction and mucosal edema characteristic of the EAR, the LAR is associated with the recruitment and activation of many cell types including eosinophils, activated $CD4^+$ T cells and activated macrophages.[74-79] Subsequent mediator release and tissue damage following these events is thought to account for the ensuing airway obstruction and associated increase in bronchial hyperresponsiveness (BHR) which accompanies the LAR. The role of the MC as a source of bronchospastic mediators during the LAR is more difficult to define than

during the EAR due to the recruitment and activation of many cell types, but the LAR is also attenuated markedly by anti-IgE therapy,[72] indicating that MC activation during the EAR initiates events leading to the LAR. It is therefore likely that the secretion of MC mediators and cytokines orchestrate the development of the LAR.

Mast Cells are Chronically Activated in Asthmatic Airways

Of particular relevance to chronic everyday asthma, MCs in asthmatic airways exhibit features of chronic on-going activation (summarized in Table 4). There are increased numbers of MCs in BAL fluid from stable asthmatics compared to normal controls,[57,80,81] together with increased concentrations of histamine and tryptase, providing evidence of on-going MC degranulation.[57,58,82] MCs from symptomatic asthmatic subjects exhibit greater constitutive histamine release in BAL and the secretory response of asthmatic BAL MCs to IgE-dependent activation is altered, with reports of increased IgE-dependent histamine release from atopic asthmatic BAL MCs compared to non-atopic normal controls.[81,82] Strong correlations have been observed between the severity of BHR and MC numbers, histamine concentrations and spontaneous histamine release in BAL fluid.[57,81,83,84] There is morphological evidence of continuous degranulation as assessed by microscopy in the airway epithelium, submucosa and smooth muscle in asthma.[85-87] Finally, there is increased protein expression and transcription for the cytokines IL-4 and IL-5 in asthmatic compared to normal bronchial mucosal MCs, again suggesting on-going activation.[88-90] While most of the studies referred to above have been performed in atopic individuals, similar evidence supports a role for on-going MC activation in both non-atopic and occupational asthma.[90-95]

Mast Cell Relocation to Airway Structures is a Key Event in Asthma Pathogenesis

It has been a long held view that the disordered airway physiology and airway wall remodeling characteristic of asthma are consequences of eosinophilic airway inflammation. However, this view is no longer sustainable. Anti-IL-5 therapy depletes airway eosinophils by over 50% and while it reduces the rate of severe asthma exacerbations,[117] it has no effect on day-to-day symptoms, BHR or lung function. There are also many examples where the relationship between eosinophilic inflammation and disordered airway physiology is weak. This is most evident in patients with eosinophilic bronchitis (EB), a condition which accounts for about 15% of cases of cough referred to respiratory specialists. It is characterized by a corticosteroid responsive cough and the presence of a sputum eosinophilia occurring in the absence of variable airflow obstruction or BHR.[118] Interestingly the immunopathology of asthma and EB in the airway mucosa are virtually identical with respect to T-cell infiltration, eosinophil infiltration and activation, mucosal MC numbers, IL-4 and IL-5 cytokine expression, epithelial integrity, sub-basement membrane collagen deposition and concentrations of histamine and PGD_2.[119-121] This suggests that many immunopathological features previously believed to contribute to asthma may not, in fact, be important in the development of airflow obstruction and BHR.[119]

MC numbers are not increased in the bronchial lamina propria of asthmatics compared to healthy subjects (Table 4). However, they infiltrate 3 key sites: the ASM[98] (Fig. 2), the airway mucous glands,[96] and the bronchial epithelium.[88] For the ASM and glands this is relatively MC-specific and places activated MCs within these dysfunctional airway elements. This is likely to have profound effects on airway physiology (see below).

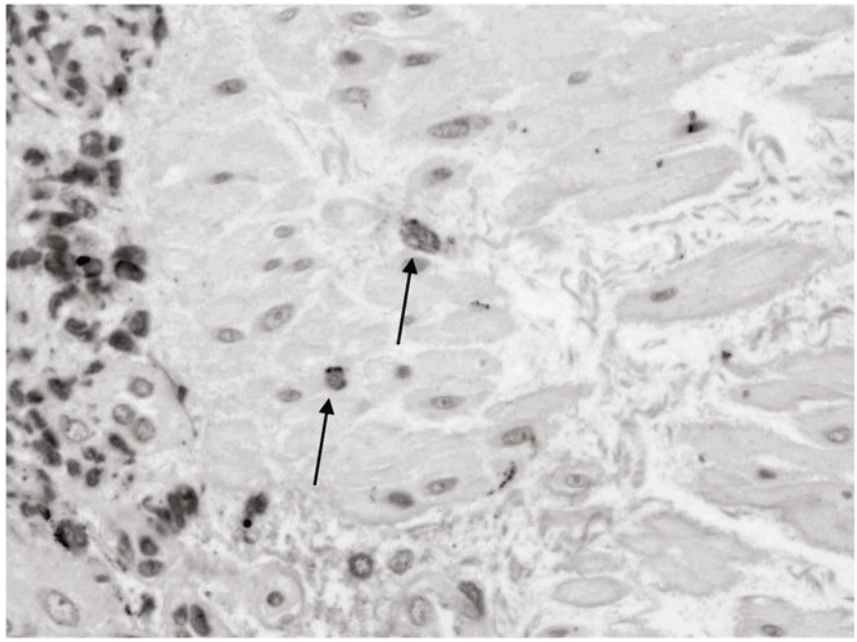

Figure 2. Mast cell infiltration of the airway smooth muscle in a patient with steroid-naïve asthma. Mast cells (arrowed) were immunostained for tryptase. Magnification x400.

Mast Cell Microlocalization within Asthmatic Airway Smooth Muscle

The striking difference between the pathology of asthma and EB lies in the ASM. Abnormal ASM function is critical in the pathophysiology of asthma and yet this structure had been largely neglected in previous immunopathological studies. In asthmatic subjects, there are numerous MCs within the ASM bundles, but virtually none in ASM from patients with EB or healthy subjects (Fig. 2).[98] This is evident across disease phenotypes such as eosinophilic and non-eosinophilic subjects and across the spectrum of asthma severity.[105,122] These ASM cells are activated and there is a correlation between the number of MCs within the ASM and the severity of BHR,[98,105,123] suggesting that this is functionally relevant. The majority of MCs within the ASM are of the MC_{TC} phenotype containing both tryptase and chymase and express IL-4 and IL-13 but not IL-5.[98,101] In contrast we found that there were almost no T cells or eosinophils in the ASM in any of the study groups. A further interesting finding is that the number of degranulated MCs within the ASM is increased in fatal compared to nonfatal asthma,[96] implicating MCs in the pathogenesis of asthma death. Interestingly, both MC numbers in the ASM and disease duration (e.g., prolonged MC presence within ASM) seem to contribute to BHR in patients.[105] Taken together, these observations indicate that ASM infiltration by MCs is a critical determinant of the asthmatic phenotype and have thus created a major paradigm shift in our understanding of asthma pathophysiology. However, this also has further implications. For example, not only might it clarify why many atopic patients do not have asthma, it would also explain why the presence of asthma is such a strong risk factor for death from anaphylaxis and allergen desensitization.[124]

Putative Mast Cell-Airway Smooth Muscle Interactions

MCs produce many mediators that adversely affect ASM function (Tables 1-3). In many instances, cellular communication within the airways is likely to work across a distance of one or two microns rather than the thousands of microns involved in the depth of the lamina propria. The microlocalization of MCs within the ASM would therefore undoubtedly facilitate specific interactions between MCs and ASM in terms of localized mediator release and direct cell-cell contact. It is therefore entirely plausible that the presence of MCs within the ASM in asthma contributes to the development of ASM hypertrophy and hyperplasia, BHR and variable airflow obstruction.

The ASM secretes many chemokines and growth factors which exhibit MC chemotactic activity, including CCL11, CXCL8 and CXCL12.[11] However, the dominant ASM-dependent chemotactic pathway for HLMCs involves the CXCR3-CXCL10 axis. HLMCs express CXCR3 and cultured ASM from asthmatic subjects preferentially secretes the CXCR3 ligand CXCL10.[5] The relevance of this to MC recruitment by the asthmatic ASM in vivo is demonstrated by the increased expression of CXCL10 by the ASM in bronchial biopsies from asthmatic compared to normal subjects and by the enrichment of CXCR3+ MCs within the ASM bundles compared to the surrounding airway mucosa.[5]

The classical MC autacoid mediators histamine, PGD_2 and LTC_4 are all potent bronchoconstrictors. In mice, instillation of Th2 cell conditioned medium to the airways of naïve animals induced BHR within 6 hours.[125] This required expression of the IL-4 receptor alpha subunit and STAT6, suggesting a critical role for IL-4 and/or IL-13. We could not find any T cells within the ASM in asthma,[98] but ASM MCs do express both IL-4 and IL-13.[101] Exogenously administered tryptase induces bronchoconstriction and BHR in dogs and sheep.[126,127] In vitro, tryptase potentiates the contractile response of sensitized bronchi to histamine and induces proliferation of human ASM, but coculture of either resting or IgE/anti-IgE activated HLMCs with human ASM cells did not induce ASM proliferation.[128-130] However, tryptase derived from HLMCs in coculture with human ASM induces the release of TGFβ from ASM cells.[131] This in turn increases the expression of α-smooth muscle actin in ASM cells, leading to increased histamine-dependent ASM contractility in collagen gels. These studies thus highlight several mechanisms through which MCs could exacerbate BHR and promote bronchoconstriction.

Contribution of MCs to local production of renin/angiotensin (Ang) in lungs has been recognized recently. Not only can MCs produce all components of a nontraditional renin-Ang system, including angiotensinogen, renin (which cleaves angiotensinogen into Ang I), chymase (which further converts Ang I into Ang II) and Ang II, but according to the data published by Veerappan et al, MCs are essential for local production of Ang II, which is a critical factor governing bronchoconstriction.[132] In isolated bronchial rings in animal models, local generation of Ang II by MC renin elicits ASM contraction mediated by Ang II Type 1 receptors.[132] Whether the components of this system are altered in asthmatic MCs and particularly those located within ASM remains to be determined.

The majority of MCs (>80%) in the ASM compartment belong to MC_{TC} subtype.[98] Chymase degrades human ASM ECM and inhibits T-cell adhesion to ASM, which might explain the paucity of T cells within this structure in asthma.[133] Also of interest, a recent mouse model has suggested that mouse MC protease 4 (a homologue of human chymase) may actually offer protection against experimental antigen-induced airway inflammation.[134] In this model, MCs were found in close proximity to ASM and absence

of MCP-4 resulted in ASM thickening in response to the OVA sensitization and challenge, clearly indicating a positive role MCP-4 in ASM remodeling.

The communication between ASM cells and HLMCs is clearly bi-directional. In coculture HLMCs adhere to human ASM cells in part via cell adhesion molecule 1 (CADM1).[135] HLMCs in coculture with ASM not only survive in the absence of exogenous survival factors, but proliferate rapidly.[136] This is mediated through a co-operative interaction between CADM1, membrane-bound SCF expressed on ASM cells and soluble IL-6. Interestingly there is evidence for physical interaction in MCs between CADM1 and KIT, suggesting that CADM1 plays an important role in KIT function.[136] Our hypothesis is that the initial adhesion mediated through CADM1 facilitates the interaction of MC KIT with ASM membrane-bound SCF. Another feature of this interaction is that HLMCs exhibit increased constitutive histamine release but can still respond to IgE/anti-IgE activation.[136] This indicates the presence of an ASM-dependent mechanism for the activation of HLMCs and could explain the presence of chronically activated MCs within the ASM bundles in asthma.

In summary, the infiltration of the ASM by MCs in human asthma is a unique feature of asthma and likely to have a central role in the pathophysiology of ASM dysfunction. There is bi-directional cross-talk between these two cell types, with evidence to suggest the presence of a positive feedback loop between MC activation and disordered ASM physiology, leading to amplification of the airway inflammatory response and BHR (Fig. 3).

Mast Cell Microlocalization within Airway Epithelium and Submucosal Glands

MCs infiltrate the bronchial epithelium in asthma.[87,88,137] This is likely to be of importance in disease pathogenesis for two reasons. Firstly, this places MCs at the portal of entry of noxious stimuli such as aeroallergens, which would facilitate an effector role in the on-going immunological response (antigen presentation, Th2 cell differentiation, IgE synthesis). Furthermore, since tryptase degrades respiratory allergens and MC IgE on FcεRI is cross-linked by allergen which may be followed by tryptase release, there may be a MC-dependent negative feedback mechanism controlling allergic inflammation.[138,139] Secondly, there are likely to be important consequences of MC activation on epithelial function. For example, MCs adhere avidly to bronchial epithelial cells and tryptase stimulates airway epithelial IL-8 release and ICAM-1 expression.[140,141] However, coculture of MCs with the BEAS-2B bronchial epithelial cell line actually inhibits IgE-dependent HLMC degranulation.[142] Whether this extends to chemokine and cytokine release is not known and awaits further work. One explanation is that normal airway epithelium keeps MCs "in check", but once the epithelium is injured, this inhibitory activity is lost and MCs are then "free" to respond to the tissue insult.

Severe mucus plugging is a well known feature of severe fatal asthma, but is also recognized as a feature of milder disease,[143] and occurs as a result of mucus hypersecretion by hyperplastic submucosal glands and epithelial goblet cells. Like the ASM, mucous glands have been largely ignored with respect to their infiltration by inflammatory cells. We had noted previously that IL-6$^+$ MCs appeared abundant amongst airway mucosal glands.[88] However, the work of Carroll and coworkers has provided strong evidence that MCs make a significant contribution to glandular dysfunction in asthma. They performed a detailed analysis of cartilaginous airways in post mortem lung specimens from patients with fatal asthma, patients with asthma who died from other causes (nonfatal asthma) and subjects without asthma who died of nonpulmonary causes.[106] The number of MCs

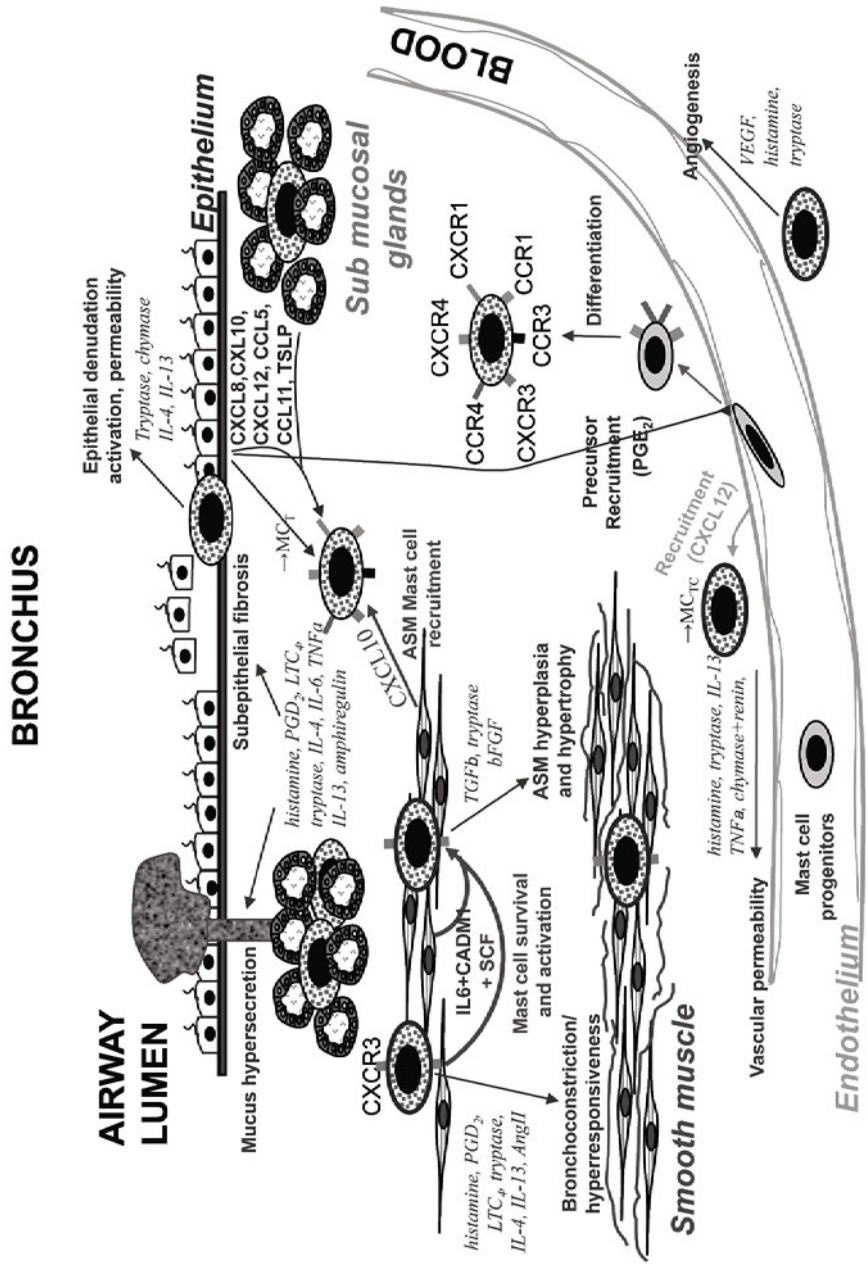

Figure 3. Summary of important interactions between mast cells and structural airway cells in asthma.

identified by tryptase immunostaining was increased significantly within the mucosal gland stroma in nonfatal asthma and a marked increase in the number of degranulated MCs was present in both fatal asthma and nonfatal asthma compared to normal controls. Importantly, the density of both intact and degranulated MCs within the mucous glands correlated strongly with the degree of mucus obstruction in the airways, again implicating MCs in the pathogenesis of asthma death. Numerous MC mediators affect glandular growth and secretion including histamine, PGD_2, LTC_4, IL-6, TNFα and chymase.[53]

Amphiregulin, a member of the epidermal growth factor family, is a further MC-derived molecule of particular interest with respect to the airway epithelium and mucosal glands. Amphiregulin expression is upregulated in human MCs following activation via IgE/anti-IgE,[103,144] and this is not inhibited by dexamethasone. MCs resident in the asthmatic bronchial mucosa demonstrate increased amphiregulin expression; and in vitro, MC-derived amphiregulin increases mucin gene expression in the NCI-H292 epithelial cell line.[103] Amphiregulin is present in increased concentrations in asthmatic sputum during asthma exacerbations; and it is proposed that this may aggravate airway wall remodeling.[145] These observations suggest that MC-derived amphiregulin may play an important role in goblet cell hyperplasia and mucus hypersecretion in asthma and that this is resistant to the effects of corticosteroids. In addition, recombinant amphiregulin induces the proliferation of human airway fibroblasts but not ASM cells, suggesting a further mechanism whereby MCs can contribute to subepithelial fibrosis. Figure 3 summarizes some potential interactions between MCs and structural airway cells in asthma.

Mechanisms of On-Going Mast Cell Activation in Asthma

The mechanisms of chronic MC activation in asthma are not fully understood. It is often assumed that allergen is the dominant factor resulting in cross-linking of FcεRI, but although allergen exposure is strongly linked to the development of asthma, once established, allergen avoidance usually has a minor effect on the state of established disease which appears to become "self-perpetuating". This is most evident in cases of occupational asthma, where asthma and accompanying MC activation will persist on removal of the sensitizing agent if exposure is not prevented early in the course of the disease. MCs can be activated by a number of diverse stimuli including monomeric IgE alone, proteases, cytokines (e.g., SCF, TNFα, IFNγ), complement, adenosine, TLR ligands, neuropeptides, hyperosmolality and cell contact (Fig. 1). It is likely that many of these pathways interact in the complex inflammatory milieu of the asthmatic airway.

Monomeric IgE induces rodent MC mediator release and prolongs MC survival through the autocrine release of cytokines.[146-149] In HLMCs IgE alone induces the release of histamine, LTC_4 and IL-8 and prolongs MC survival after the withdrawal of SCF and IL-6 through autocrine secretion of IL-6.[150,151] Importantly, while free IgE is present, intracellular signaling continues, suggesting that these findings are physiologically relevant. This is interesting because in humans there is a reproducible correlation between total serum IgE concentration, BHR and asthma.[152-155] Thus, heightened MC activation may arise in part from the increased binding of IgE to FcεRI. This is supported to some extent by the observation that anti-IgE therapy markedly reduces both airway inflammation and MC activation as manifest by reduced IL-4 expression.[156] However, anti-IgE therapy has a minimal effect on BHR, suggesting that monomeric IgE-dependent activation of MCs in asthmatic ASM does not account for this aspect of airway dysfunction.

SCF primes MCs for mediator release and at higher concentrations, directly induces degranulation.[157] Concentrations of SCF are elevated in asthmatic airways and could therefore make an important contribution to ongoing MC activation.[158,159] Interestingly, SCF and IgE have an important effect on the efficacy of β_2-adrenoceptor agonists, used as reliever medication for asthma, with respect to HLMC function. When applied to HLMCs acutely in vitro in the absence of SCF, they inhibit IgE/anti-IgE-dependent degranulation.[69] However, after sustained exposure to the drug, rapid desensitization occurs with loss of efficacy.[160] Similarly, in vivo, acute administration of salbutamol and the long-acting β_2-adrenoceptor agonist salmeterol to asthmatic subjects inhibit the airway response to allergen challenge. However, if these drugs are administered regularly for 1 week, not only is the protection against the EAR lost, the magnitude of the EAR and the accompanying MC mediator release are enhanced.[161,162] Furthermore, regular administration of short-acting β_2-adrenoceptor agonists to asthmatic subjects has been associated with loss of asthma control.[163,164] When HLMCs are incubated with SCF, their ability to respond to β_2-adrenoceptor agonists is lost within 2 minutes.[165] This is caused by the rapid uncoupling of the β_2-adrenoceptor in HLMCs due to phosphorylation of Tyr350 on this receptor and subsequent internalization. Furthermore, in the presence of both SCF and IgE, salbutamol produced a dose-dependent increase in histamine release. This mechanism might explain the above clinical findings. If this applies in vivo, then targeting SCF may enhance the efficacy of β_2-adrenoceptor agonists and improve asthma control. Further evidence that this might occur in vivo comes from a study of asthma death, which demonstrates that the extent of MC degranulation correlates with blood salbutamol concentrations.[166]

The C5a receptor CD88 is expressed on the MC_{TC} subset of HLMCs.[10] Elevated C5a concentrations have been identified in the induced sputum of asthmatic subjects,[167] thus providing a further potential means of MC activation and of particular relevance to those MCs (MC_{TC}) within the ASM bundles.

As described above, HLMCs express the TLR-1-5, -7 and -9,[47] stimulation of which results in MC activation. Perhaps of most relevance to asthma is MC activation via TLR3 with double-stranded viral RNA ligand. Poly I:C, a synthetic activator of TLR3, induces the specific release of IFNα as does exposure to RSV and influenza virus.[35] Since viruses are a common cause for asthma exacerbations, the MC antiviral response may be an important contributor to the deteriorating airway physiology. Nakayama and co-authors investigated the role of TLR4 on MCs in allergic airway inflammation using MC-deficient $Kit^{W/Wv}$ mice and $TLR4^{-/-}$ mice in an asthma model.[168] Ovalbumin-induced eosinophilic inflammation in the lung was dramatically increased by the administration of LPS in wild-type mice, whereas the same increase was not observed in MC-deficient or TLR4-deficient mice. Adoptive transfer of bone marrow-derived MCs from wild-type, but not from TLR4-deficient mice restored the increased eosinophilic infiltration in the airway in MC-deficient mice.[168] LPS inhalation increased IL-5 production by lung MCs and exacerbated airway inflammation in mouse asthma model.[169] In vitro studies revealed that LPS treatment augmented levels of IL-5 and -13, as well as mRNA expression of IL-4, IL-5 and IL-13 and CCL24 (eotaxin-2) in wild-type bone marrow MCs, whereas no increase in IL-5 and eotaxin-2 was detected the case of $TLR4^{-/-}$ MCs.[168] Interestingly, production of IL-6 and TNF-α was not significantly changed by LPS in wild-type MCs. Moreover, cotreatment of bone marrow MCs with LPS with IgE/antigen showed synergistic effects on the expression of IL-5 and -13 and CCL24. The TLR4 mediated signaling involved immediate NF-kappaB activation, followed

by increase in the levels of GATA1 transcription factor responsible for activation of the IL-4, IL-5 and IL-13 promoters. Furthermore, examination of the gene expression profile from human peripheral blood MCs using high density oligonucleotide probe arrays following activation with LPS via TLR4 compared to anti-IgE demonstrates that both induce a core response, plus an LPS or anti-IgE specific program of gene expression.[170] LPS-stimulated MCs specifically induced a subset of genes that included a Th2 cytokine and chemokines that recruit Th2 cells and eosinophils.[170] These data indicated that TLR4 in MCs may be involved in LPS-mediated enhancement of allergic airway inflammation.

MC TLRs also have the potential to improve asthma. Pretreating mice with the TLR9 ligand, immunostimulatory sequences (ISS) of DNA containing a CpG motif, significantly inhibited the accumulation of peribronchial MCs and the expression of IL-4 and IL-9 in lung, as well as reduced the increased airway hyperreactivity to methacholine; all caused by chronic antigen challenge.[48] In vitro experiments on MCs derived from the bone marrow of these mice showed that ISS did not affect histamine release, but induced IL-6 production.[48] These data suggest that stimulation of TLR-9 may have beneficial effect in asthma model.

Thymic stromal lymphopoetin is a cytokine released from airway epithelial cells in response to injury.[171,172] Its overexpression in mice leads to asthmatic-type Th2-driven airway changes, while its inhibition attenuates the airway changes seen in mouse models of asthma.[173] One paradigm for the effects of TSLP is that it activates CD11c+ dendritic cells which in turn leads to the differentiation of $CD4^+$ T cells into the Th2 phenotype through an interaction of OX40 with its ligand.[174,175] However, it is well documented that T-cell-independent pathways also contribute to TSLP-dependent inflammation. In particular, the direct interaction of TSLP with MCs is highly relevant to the development of allergic-type airway inflammation. TSLP, derived from human epithelial cells activated with cytokines, peptidoglycan or trauma, induces the release of numerous cytokines and chemokines from human MCs, including IL-1.[172] IL-13 production by MCs is inhibited when endogenous TSLP released by activated human epithelial cells is blocked.[172] Furthermore, in a mouse model of rhinitis, MC activation was required for the increased TSLP production by airway epithelium.[176] MC-derived TSLP also has the potential to contribute to the development of airway inflammation.[104] TSLP is overexpressed in asthmatic airways,[177,178] and so both MC-derived and epithelial-derived TSLP may be a key cytokine driving the MC in asthma.

Immunoglobulin (Ig) free light chains are present in serum in normal subjects and their production is augmented in inflammatory diseases such as rheumatoid disease. In mice Ig free light chains can confer MC-dependent hypersensitivity through an unknown mechanism,[179] and antigen-specific light chains can mediate MC-dependent bronchoconstriction following antigen challenge.[180] Concentrations of Ig free light chains are elevated in the sera of asthmatic compared to normal subjects suggesting they may be relevant to the pathophysiology of human asthma.[180]

Genetic factors may also lower the threshold for MC activation in asthma and thus predispose to the development of the disease and/or its severity. For example, an important negative regulator of MC activation is the phosphatase SHIP.[181] SHIP-deficient MCs exhibit markedly enhanced secretory responses and with respect to human basophils at least, cells that are "hyperreleasable" demonstrate a relative deficiency of this molecule.[182]

Mast Cells as Initiators of Allergic Airway Inflammation

Conventionally, MC activation in asthma has been interpreted as an IgE-dependent event, but recent studies indicate that common allergens, many of which are serine proteases, can induce MC histamine and cytokine secretion directly through an IgE-independent mechanism.[183,184] This suggests that MCs may not only contribute to the chronic airway inflammatory response and symptomatology, but may have a central role at the initiation of the allergic immune response, i.e., providing signals inducing B-cell IgE synthesis and Th2 lymphocyte differentiation.[185,186]

Mast Cells in Animal Models of Asthma

Several models have been developed which aim to induce the airway features of asthma. The most widely reported is the mouse model using intraperitoneal antigen sensitization followed by antigen challenge of the airways. This most closely resembles the model of acute allergen challenge in the airways although the route of sensitization is obviously different. The dependency on MCs in these models with regard to the development of airway hyperresponsiveness and inflammatory changes in the airways is highly dependent on the model studied and the mode of antigen sensitization. Thus sensitization without adjuvant generates a MC-dependent model while sensitization with adjuvant creates a MC-independent model.[187]

An alternative model uses airway sensitization without adjuvant from the outset and, to some extent, this is more physiological. In this setting, MCs are again an essential component required for the development of airway hyperresponsiveness, inflammation and remodeling (collagen deposition, goblet cell hyperplasia.[188-190] Furthermore, mouse models allowed to estimate contribution of infections and TLR activation, as well as the nervous system[42,168,169,191,192] to MC function in the development of asthma. However, there are inevitably a number of problems in relating these models to the human disease. For example, mouse airways contain very few MCs at baseline, so the changes seen following antigen challenge rely heavily on the recruitment of MC progenitors rather than the activity of resident cells. Therefore, it is perhaps not surprising that short term models using intraperitoneal sensitization do not reveal a role for MCs in the outcomes commonly measured. In addition, mice have relatively little smooth muscle in their airways and so there is no model described to date in mice which has recapitulated the infiltration of ASM by MCs; a feature that may be key to the development of asthma in humans. So while mouse models are useful for generating hypotheses regarding the pathogenesis of asthma, their findings may also be potentially misleading.

Mast Cells in Chronic Obstructive Pulmonary Disease (COPD)

COPD, one of the leading causes of death worldwide, is characterized by airflow limitation that is largely fixed and which is usually progressive. This is associated with an abnormal inflammatory response of the lungs to noxious particles or gases, e.g., tobacco smoke. Inflammation is followed by scarring and remodeling with airway thickened walls; the latter is followed by fibrosis and destruction of alveoli. In contrast to asthma where there is overwhelming evidence for MC involvement, their role in COPD is less obvious (Table 4). In COPD patients, the MC population in the lung undergoes changes in density and distribution related to a severity of the disease. In contrast to asthma, the total MC

density in lung, as well as MC_T in submucosa, progressively decreases compared with healthy lung, but MC activation and degranulation are increased as in asthma, coinciding with enhanced histamine release in advanced COPD.[107,193] Similar to asthma, an increased MC_{TC} density has been reported in alveolar parenchyma and in ASM.[107,109] In lungs from patients with very severe COPD, there is a several-fold increase in the MC_{TC}/MC ratio cells in small airways, small airway epithelium, pulmonary vessels and alveolar parenchyma, with MC_{TC} comprising ~95% of the total MCs in larger airways.[107] Of particular importance is the increased CD88 expression in both MC_{TC} and MC_T cells,[107] which coincides with increased expression of the CD88 ligand c5a in COPD patients[167] and which is likely to cause MC activation. The changes in MC_{TC} population also coincide with increased expression of TGFβ and renin, but their functional importance in COPD has not been elucidated. Interestingly, increased MC_T and MC_{TC} numbers positively correlate with the lung function in COPD.[109] High levels of MC activation in COPD are also likely to be caused by elevated bronchial mucosal expression of TSLP and chemokines similar to that found in severe asthma.[177]

Mast Cells in Interstitial Lung Disease

Interstitial lung diseases are characterized by the presence of pulmonary fibrosis occurring as a result of injury and aberrant repair. The most common form of pulmonary fibrosis encountered in the clinic is idiopathic pulmonary fibrosis (IPF) with a histological pattern of usual interstitial pneumonia (also known previously as cryptogenic fibrosing alveolitis) (reviewed in see ref. 194). While the initiating events and role of inflammation in the onset of IPF remain controversial, there is good evidence of ongoing damage to the alveolar epithelium, basement membrane and capillary endothelium, leading to the activation of repair mechanisms with infiltration of fibroblasts, MCs and other inflammatory cells. The chronicity of the insult leads to dysregulated repair with the generation of fibroblastic foci. These events are driven by several profibrotic mediators and cytokines including TGFβ1, PDGF and bFGF.[194] The key cell driving the development of fibrosis is the myofibroblast, which has an intermediate phenotype between fibroblasts and smooth muscle, in that it expresses α-smooth muscle actin (αSMA) and exhibits contractile activity, but is capable of the synthesis and deposition of extensive fibrotic matrix.[195] The myofibroblast is therefore a highly attractive target for the treatment of IPF.

In fibrotic lung disorders, there is an increased MC number in lung tissue compared with the control group, with MC counts significantly correlating with the degree of fibrosis (Table 4).[110] In lungs of patients with idiopathic bronchiolitis obliterans organizing pneumonia, MC density and activation are also increased.[112] In addition increased MC density, with MC_{TC} correlated to accumulation of ASM cells and myofibroblasts, is found in idiopathic interstitial pneumonia.[113] In both normal and fibrotic lung, the majority of MCs and fibroblasts in the alveolar septae are apposed.[196] A marked increase in MC density and MC hyperplasia has been described in the thickened fibrous alveolar septae, as well as in MCs also were found within the alveolar epithelial layer and alveolar lumina of patients with pulmonary fibrosis resulting from several diverse etiologies in contrast to control specimens, in which MCs were most frequently seen in subpleural and perivascular connective tissue.[111] These MCs often show reduced numbers of granules and disorganized granule content, suggesting that there is partial ongoing degranulation. In addition, about 10-fold-elevated histamine concentrations have been described in BAL from patients with IPF.[197] A specific feature of IPF is the abundant bFGF-expressing cells in lung

interstitium, which have been identified as MCs.[114-116] These bFGF-containing MCs are surrounded by collagen, elastic fibers and smooth muscle cell/myofibroblast-like cells.[116] Furthermore, the distribution of bFGF-containing MCs matches that of ECM deposition and correlates with the extent of fibrosis; morphometrically, providing circumstantial evidence that MCs contribute to the fibrotic process.

Alveolar hypoxia reportedly induces activation of MCs, leading to local generation of Ang II and consequently to systemic inflammation.[198] MC stabilization with cromolyn prevented the systemic response, as did ACE inhibitors and AngII receptor blockers, through interactions between leukocytes and endothelial cells. It is conceivable that similar mechanisms may activate MCs in pulmonary fibrosis and link MC activation with systemic inflammation in COPD. Fibroblasts/myofibroblasts interact intimately with MCs through cell-cell adhesion thereby inducing the release of various MC-derived mediators. For example, cultured rat embryonic skin fibroblasts phagocytoze rat MC granules and this is followed by secretion of collagenase and β-hexosaminidase.[199] Histamine, TNFα, bFGF, TGFβ and IL-4 promote fibroblast proliferation in humans.[200-204] In coculture, human MCs adhere avidly to human fibroblasts through undefined receptors.[205] MCs augment proliferation of human skin fibroblasts via MC-derived IL-4, independently of cell-cell contact.[206] However, IL-4 could not be detected in coculture, suggesting that IL-4 was secreted by MCs in low amounts and strictly limited to cell-cell contacts with fibroblasts. We proposed that MCs may present IL-4 on their surface in order to confer local cytokine specificity.[207] IL-4 is a chemoattractant for human fibroblasts and also induces human fibroblasts to secrete ECM proteins.[208,209]

Among HLMC-derived mediators, bFGF is likely to have a major effect on lung fibroblasts. Heparin stabilizes bFGF structurally and preserves its bioactivity by protecting it from degradation.[210] Furthermore, heparin and/or heparan sulfate are required for binding of bFGF to its receptors[211] and also release it from basement membranes where it is stored.[212] Heparin may thus potentiate fibroblast activation and proliferation indirectly through the regulation of bFGF activity. In addition, MC tryptase induces proliferation of lung parenchymal and airway fibroblasts via activation of PAR-2.[213]

In animal models, increased numbers of MCs and elevated lung histamine content have been documented in pulmonary fibrosis induced by bleomycin, radiation and asbestos.[214-216] In the murine model of silica-induced pulmonary inflammation, MC hyperplasia is also seen, but MC-deficient mice develop significantly less severe lung lesions and have a reduced BAL neutrophilia and protein content.[217]

MCs are also implicated in pulmonary capillary hemangiomatosis, causing pulmonary arterial hypertension with no effective medical therapy. Microarray analysis of lesions from patients with this rare disease has identified overexpression of MC-related genes for surfactant protein C and tryptase.[218] MCs, identified by CD117 immunostaining, confirmed an abundance of MCs with high PDGFR-β expression in this disease.

Lung Cancer

The role of MCs in cancer is discussed in more detail in Chapter 14. Lung cancer is the leading cause of death from malignancy worldwide with nonsmall cell lung cancer (NSCLC) accounting for the majority of these cases. There is extensive evidence indicating that the immune system plays a key role in the regulation of cancer development and progression,[219] and cells of the innate and adaptive immune responses have been implicated in both the progression and curtailment of tumor growth.[220] The role of MCs in tumor

development and progression is controversial due to the nature of the mediators they generate.[221] Several lines of evidence have suggested that MCs promote tumor progression and metastasis,[222-224] potentially through their ability to promote angiogenesis via the release of autacoid mediators and pro-angiogenic chemokines and growth factors.[225,226] For example, the granular products of MCs, histamine and cannabinoids released during degranulation, have been demonstrated in coculture to enhance the migration of cervical cancer cells.[227] Recently a peptide adrenomedullin implicated in cross-talk between MCs and cancer cells was localized in MCs within lung cancer.[228]

Increased MC numbers have been documented to correlate with poor outcome in patients with NSCLC, especially lung adenocarcinoma.[222-224] and microvessel density,[222-224,229-233] although exceptions have been noted.[234,235] Nevertheless, other studies found positive correlations between MC intratumoral density and good prognosis,[230,236,237] particular at early stages and specifically for the MC_{TC} type.[233,234] MC counts also negatively correlate with the presence of metastatic lung cancer in lymphatic nodes.[238] There are however reports of lack of correlation between lung cancer survival and MCs.[232,235,239] Regardless of their findings, a problem with these studies is that none of them paid attention to the location of MC within NSCLC tumors. We therefore carefully examined the density of tryptase+ MC in the tumor stroma compared to the clusters of tumor islets in 175 patients with surgically resected NSCLC. These studies revealed that MC numbers were similar in the tumor stroma of patients with surgically resected NSCLC irrespective of survival status, but there was a marked survival advantage when MC were present in NSCLC tumor islets.[237] Therefore, as in other locations and diseases, the microanatomical location of MC is likely to be key with respect to their biological activity within the tumor microenvironment.

CONCLUSION

There is overwhelming evidence, based largely on studies from humans, that MCs play a central role in asthma pathogenesis and are undoubtedly involved in many diverse pulmonary diseases. The goal of current research is to understand the mechanisms driving their chronic activation and cellular cross-talk, with a view to developing novel therapies which are effective when administered to patients long-term in vivo.

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CHAPTER 14

MAST CELLS, ANGIOGENESIS AND CANCER

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Abstract: Mast cells (MCs) were first described by Paul Ehrlich[1] in his doctoral thesis. MCs have long been implicated in the pathogenesis of allergic reactions and certain protective responses to parasites. As most tumors contain inflammatory cell infiltrates, which often include plentiful MCs, the question as to the possible contribution of MCs to tumor development has progressively been emerging. In this chapter, the specific involvement of MCs in tumor biology and tumor fate will be considered, with particular emphasis on the capacity of these cells to stimulate tumor growth by promoting angiogenesis and lymphangiogenesis. Data from experimental carcinogenesis and from different tumor settings in human pathology will be summarized. Information to be presented will suggest that MCs may serve as a novel therapeutic target for cancer treatment.

INTRODUCTION

Mast cells (MCs) are versatile, tissue-homing secretory cells, which were first described by Paul Ehrlich[1] in his doctoral thesis.[2] MCs have long been implicated in the pathogenesis of allergic reactions and certain protective responses to parasites. They have been linked to various cell-mediated immune reactions, being found in tissues from multiple disease sites and as a component of the host reaction to bacterial, fungal and viral infections. They have also been shown to participate in angiogenesis and tissue repair processes after injury.[3,4]

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The importance of a possible functional link between chronic inflammation and cancer has long been recognized. As most tumors contain inflammatory cell infiltrates, which often include plentiful MCs, the question as to the possible contribution of MCs to tumor development has been emerging. A major point linking MCs to cancer is the well recognized capacity of these cells to synthesize and release potent angiogenic compounds. Although some evidence suggests that MCs can promote tumorigenesis and tumor progression, there is some clinical evidence, as well as experimental tumor models, where MCs seem to have functions that favour the host. Thus, the involvement of MCs in tumor development is complex and far from being settled.

We will examine the specific involvement of MCs in tumor biology and tumor fate, with particular emphasis on the capacity of these cells to stimulate tumor growth by promoting angiogenesis and lymphangiogenesis. Then, data from experimental carcinogenesis and from different tumor settings in human pathology will be reviewed. The last section will be dedicated to drugs affecting MC number.

MAST CELLS IN EXPERIMENTAL CARCINOGENESIS

Involvement of MCs in experimental carcinogenesis has increasingly attracted the interest of researchers. Development of squamous cell carcinoma in a HPV (human papilloma virus) 16-infected transgenic mouse model of epithelial carcinogenesis has provided experimental evidence in favour of an early participation of MCs in tumor growth and angiogenesis.[5] Accumulation of MCs was observed at the hyperplastic and dysplastic stages and in the invasive edge of tumor growth. They were typically localized around capillaries and epithelial basement membrane, where MC degranulation resulted in the release of the MC-specific proteases, tryptase and chymase. MCs were absent within the tumor mass itself. Infiltration of MCs and activation of matrix metalloproteinase (MMP)-9 coincided with the angiogenic switch in premalignant lesions. Addition of chymase alone was sufficient to stimulate an angiogenic phenotype when coincubated with a hyperplastic skin sample in vitro, while tryptase was shown to play a prominant role in tissue remodelling.

Gounaris et al[6] developed a transgenic mouse model over-expressing an inducible, stabilized β–catenin in intestinal enterocytes, as well as the $APC^{\Delta 468}$ as an independent hereditary model of polyposis. In both mice, developing polyps were rapidly infiltrated by $CD34^{+}$ MCs; and histological staining confirmed the presence of both connective and mucosal type MCs in developing tumors. The latter MC type was prevalent at the site of newly developing polyps, while the former was prevalent in the stroma at later stages of tumor development. Treatment with antitumor necrosis factor alpha (TNFα) antibodies resulted in fewer polyps, reduced polyp growth and decreased MC infiltration. Reconstitution of W^{sh}/W^{sh} MC-deficient mice with $CD34^{-/-}$ $CD43^{-/-}$ bone marrow also resulted in a decrease in polyp growth, as compared to mice reconstituted with wild type bone marrow. Overall, these data suggest that MCs are an essential component for preneoplastic polyp development as well as a possible target for therapeutic intervention.

In a pancreatic β-cell tumor model, in vivo activation of Myc was shown to induce several chemokines in developing tumors, including chemokine ligand 2 (CCL2), monocyte chemotactic protein 1 (MCP-1), and CCL5. This latter chemokine triggered rapid recruitment of MCs to the tumor site, recruitment that is absolutely required for macroscopic tumour expansion.[7] MCs were the only inflammatory cells increased in the

vicinity of tumor cells at the early stage of tumor growth and their infiltration correlated with the expansion of islet tumors. Macrophages and neutrophils were also recruited after the onset of tumor angiogenesis. Treatment of established β-cell tumors with disodium cromoglycate (cromolyn), an inhibitor of MC degranulation, rapidly triggered hypoxia and cell death of tumor and endothelial cells. The authors concluded that MCs are necessary for tumor angiogenesis and growth; and suggested that inhibition of MC function might prove to be therapeutically useful in restraining the growth of pancreatic cancer. In an in vivo transplantation assay in nude mice, angiopoietin-1 (Ang-1) secreted by primary murine MCs promoted marked neovascularization.[8] The MCs studied were bone marrow mast cells (BMMCs) from C57BL/6 mice and two mouse plasmocytoma cell lines. Greater angiogenesis was demonstrated when BMMCs were transplanted into mice together with plasmocytoma cells, than when each of the cell types was transplanted alone. The use of Ang-1-neutralizing antibodies significantly reduced the growth of plasmocytoma containing MC. Overall, these data demonstrate that MC-derived Ang-1 promotes growth of plasmocytomas by stimulating neovascularization.

Hart et al,[9] using W/W^v mice, demonstrated a direct correlation between MC density in the dermis and susceptibility to ultraviolet-B-induced systemic immunosuppression. These mice, which are homozygous for the W (white spotting) mutation and therefore severely MC deficient, are unresponsive to ultraviolet-induced immunosuppression unless first injected with MC precursors at the irradiated site.[9] The W locus encodes the KIT tyrosine kinase receptor that binds stem cell factor (SCF).[10] The MC products involved in ultraviolet-induced immunosuppression are believed to be TNFα and histamine.[11]

These data lend support for a crucial role of MCs in early carcinogenesis in different experimental settings.

MAST CELL RECRUITMENT TO TUMOR

Recent literature identifies MCs as critical regulators of inflammation and the immunological response in the tumor microenvironment. Chronic inflammation induces both proliferation of resident tissue MCs and recruitment of MCs and their precursors from outside the lesion.[12,13] During tumor development, circulating MC precursors migrate into the neoplastic mass and form one of the major stromal cell populations. Recruitment and activation of MCs in the tumor infiltrate is mainly mediated by tumor-derived stem cell factor (SCF) through its receptor KIT on MCs.[14] In a hepatocarcinoma model, MCs failed to migrate into SCF-knockdown tumors and anti-KIT antibodies abolished the migration of MCs into tumors, leading to decreased tumor growth. Low concentrations of SCF efficiently induced the chemotactic migration of MCs and the release of active MMP-9 into the local environment. Higher SCF concentrations promoted activation of recruited MCs in the tumor context, which were induced to release pro-inflammatory factors including interleukin (IL)-6, TNF-α, vascular endothelial growth factor (VEGF), Cox-2, i-NOS and CCL-2.[14] The intervention of SCF-activated MCs in the tumor microenvironment increased the transcription of the IL-17 gene and the number of IL-17-producing cells in the tumor mass. In turn, IL-17, a potential candidate for regulating the tumor inflammatory reaction through the production of IL-9, would attract more MCs to the site of inflammation.[15] TNF-α and other pro-inflammatory factors released by MCs can similarly increase the activity of NK-κB and AP-1 in tumor cells.[14] NK-κB and AP-1 may, in turn, favour the proliferation of tumor cells by inducing the expression of cyclines, the survive of tumor

cells by the blockade of apoptosis and the invasiveness of tumor cells by inducing the production of macrophage migration inhibitory factor (MIF) and EMMPRIN.[16,17]

MAST CELLS AND IMMUNE TOLERANCE

A new paradigm of immunological participation for MCs has recently been proposed to explain their involvement in the dynamics of tumor development and progression. Although immune surveillance works at an early stage of tumorigenesis, established tumors primarily induce immune tolerance by creating sites of immune privilege and by inducing a shift of the immune balance from activation to tolerance.[18,19] MCs have recently been proposed to be mechanistically involved in the negative modulation of immune surveillance in the tumor microenvironment (Fig. 1). MCs have thus been found to play a critical role in the suppression of immune reactions.[20] These cells not only produce inhibitory cytokines, such as IL-10,[21] but also are essential in promoting the immune tolerance mediated by regulatory T (Treg) cells. Indeed, MCs serve as enforcers for Treg cells, turning down the immune system's reaction to a skin allograft, possibly through IL-10 secretion.[22] Ultraviolet-B irradiation which represents the most important skin immunesuppressor and initiator of cutaneous malignancies, activates MCs.[23,24] Upon

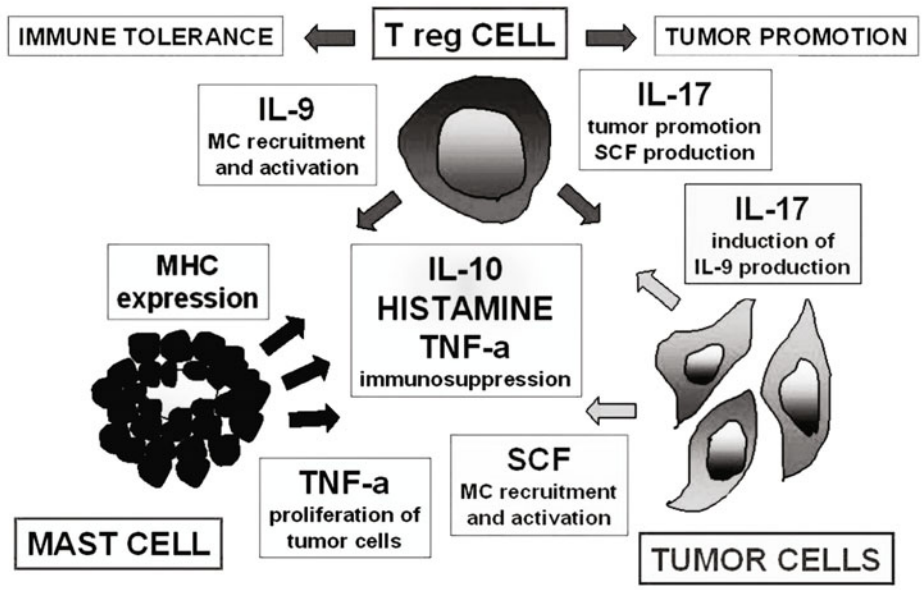

Figure 1. The immuno-suppressive role of MCs in the tumor environment. MCs infiltrating the tumor stroma favor expansion and activation of regulatory T cells which, in turn, stimulate immune tolerance and tumor promotion. SCF-mediated recruitment of MCs by tumor cells leads to MC activation and release of immunosuppressive and tumor-promoting molecules such as IL-10, histamine and TNF-α. Upon activation, regulatory T cells release SCF and IL-9, which expand the MC population. MCs serve as enforcers for regulatory T cells, possibly by MHC-mediated activation. Abbreviations: T reg cell, regulatory T-cell; SCF, stem cell factor; TNF-α, tumor necrosis factor-α; MHC, major histocompatibility complex; IL-9, IL-10, IL-17, interleukin-9, -10, -17.

irradiation of the skin, *trans*-urocanic acid in the epidermis isomerizes to *cis*-urocanic acid, which stimulates neuropeptide release from neural c-fibers. These neuropeptides, in turn, trigger secretion of histamine, TNF-α and other mediators from MCs, leading to suppression of the cellular immune system.[11,25] Using MC-deficient W/W^{v} mice, a direct correlation has been demonstrated between MC density in the dermis and susceptibility to ultraviolet-B-induced systemic immunosuppression.[9] In a skin transplantation model of allograft tolerance in the mouse, MCs were crucial for graft acceptance, as MC-deficient C57BL/6-*Kit* $^{W\text{-}sh/W\text{-}sh}$ mice showed an inability to induce tolerance.[22] Activated Treg cells in the tolerant tissue produced high levels of IL-9, a cytokine which seems important in MC recruitment, growth and activation. This cytokine appears to be a crucial factor in mediating regional tolerance, as neutralization of IL-9 greatly accelerates allograft rejection in tolerant mice.[22] In a mouse model of tumorigenesis, SCF-activated MCs exacerbated immunosuppression in the tumor microenvironment.[14] MCs were shown to promote a decrease in mRNA for the immune activating factor IL-2, while inducing an increase in mRNAs for the immune suppressor factors IL-10, transforming growth factor beta (TGF-β) and forkhead box p 3 (Foxp3) in the tumor. The percentage of Treg cells in the total T-cell number in the tumor mass was also increased because $CD4^{+}CD25^{-}$ T cells can be converted into $CD4^{+}CD25^{+}$ regulatory T cells by TGF-β induced expression of Foxp3.[26]

Another mechanism of potential physiological and pathological significance in MC-driven Treg cell expansion might be linked to the lipopolysaccharide (LPS) and interferon (IFN)-γ-induced expression of MHC class II on MCs. The expression of MHC-II grants MCs the ability to process and present antigens directly to T cells. In fact, MCs preferentially expand antigen-specific Treg cells over naïve T cells which may help explain allograft tolerance induction by MCs.[27] MCs are poor stimulators of naïve T cells in vitro. However, one consequence of MHC-II expression on MCs may be to activate regulatory Treg cells and thus dampen the immune response. Activation of Treg cells by MCs may also thus contribute to the protective effect of MCs on skin allografts.[22,27] A recent publication supports the role of MC-Treg cell interactions in promoting immunosuppression and tumor development. The density of peritumoral MCs and Treg cells was evaluated to predict the prognosis of hepatocellular carcinoma.[28] It was found that higher peritumoral MC numbers were associated with poorer clinical outcome. Peritumoral Treg cells were positively correlated with MC density and inversely related to hepatocellular carcinoma outcomes. Further, MC number in combination with Treg cell number could predict prognostic outcomes more effectively than MC numbers alone. Thus, MCs and Treg cells may cooperate with each other in hepatocellular carcinoma to result in a poorer prognosis.[28]

In conclusion, the pivotal role of MCs in the induction of an acquired condition of immune tolerance appears to be well established in various experimental settings. The secretion of immunosuppressive mediators by MCs seems to be critical for sustaining tolerance.

MAST CELLS AND TUMOR GROWTH IN HUMANS

As noted, MCs accumulate at sites of tumor growth in response to numerous chemoattractants, in particular SCF secreted by tumor cells.[14,29] MCs are endowed with a vast array of mediators, some of which have promoting and others, inhibitory effects on malignancies.[30] Degradation of the extracellular matrix (ECM) plays a critical role in tumor expansion and metastasis. Dabbous et al[31] showed that MC degranulation is associated

with disruption and lysis of the tumor ECM, either directly through the action of their enzymes, or indirectly through modulation of the collagenolytic activity of fibroblasts, macrophages and tumor cells.[32,33] Tryptase activates latent MMP and plasminogen activator, which, in turn, degrade the ECM.[32] MCs cause apoptosis in various target cells[34] and induce the accumulation of tumor associated inflammatory cells in vivo.[35]

Heparin enhances both the activity and production of collagenase in vitro and release plasminogen activator from endothelial cells.[36,37] MCs are a major source of histamine, which could induce tumor cell proliferation through H1 receptors identified in human malignant carcinoma, while suppressing the immune system through H2 receptors.[38] High histamine concentrations inhibit human primary melanoma-cell proliferation, presumably by acting through H1 receptors, an action enhanced by IL-2. Low amounts of histamine acting through H2 receptors increased this cell proliferation.[39] The incidence of metastases, as well as the appearance of tumors, correlates inversely with the tissue histamine level.[40] MCs may also exert direct mitogenic effect on tumor cells through additional mediators. For example, mast cell-derived fibroblast growth factor (FGF)-2 and IL-8 are directly mitogenic to melanocytes and melanoma cells.[41,42]

Histological examination of many kinds of tumors has identified MCs in the inflammatory infiltrate. For instance, MC density in benign gastric ulcers was found to be much higher than in control subjects[43] and MC accumulation is also increased in well-differentiated gastric cancers when compared with controls. These observations suggest a role for MCs in the shift between inflammation and cancer. However, poorly-differentiated gastric adenocarcinomas showed lower MC density than well-differentiated adenocarcinomas.[43] MCs have also been detected in bilateral multiple spindle cell lipomas of the tongue.[44] It has similarly been suggested that MCs may be responsible for a fibrotic reaction occurring in the solitary sclerotic neurofibroma of the skin.[45] By electron microscopy, MCs were seen in close contact with fibroblasts and abundant collagen fibers were noted in the lesions. MCs have been found in great numbers at the periphery and in between the tumor islands of basal cell carcinomas.[46] In addition, MCs in basal cell carcinoma have been found to express VEGF, IL-8 and CCL5 (RANTES), suggesting an active MC involvement in the tumor microenvironment.[47] An increased number of MCs has been detected in the mononuclear cell infiltrate of telangectasia macularis eruptiva perstans lesions,[48] in a case of conjunctival myxoma,[49] and in synovial sarcoma.[50]

CORRELATION WITH PROGNOSIS

In many tumor settings, the number of infiltrating MCs has been shown to correlate with the clinical tumor stage and to represent a significant prognostic factor. A progressive increase in MC number associated with a increasingly poor prognosis has been shown during the progression of human melanoma and oral squamous carcinoma.[51,52] Rojas et al[53] demonstrated a higher number of MCs in squamous cell carcinoma of the lip, as compared to normal lip. Sharma et al[54] reported a higher number of MCs in nodular sclerotic-type Hodgkin's lymphoma (HL) than in other types of HL, and the number of MCs was higher in fibrotic areas than in cellular areas. Molin et al[55,56] observed a worse prognosis for a nodular sclerosing HL exhibiting a high MC number. Fukushima et al[57] demonstrated an increased number of MCs in fibrotic areas in diffuse large B-cell lymphoma (DLCCL) lymph nodes. The greatest number of MCs among T-cell lymphomas was observed in

angioimmunoblastic T-cell lymphoma.[58] Dave et al[59] utilized gene array to study the relationship between prognosis and a specific gene expression profile and concluded that the length of survival among patients with follicular lymphoma correlated with the molecular features of nonmalignant immune cells present within the tumor at the time of diagnosis. One of the genes observed to correlate most negatively with survival was microphthalmia-associated transcription factor (MITF), a transcription factor which has been found to be highly expressed in MCs and to play a critical role in the regulation of several key MC-specific genes.[60] Tournilhac et al[61] demonstrated that MCs may support tumor cell expansion in Waldenstrom's macroglobulinemia through constitutive CD154-CD40 signalling. Moreover, the use of a CD154-CD40 signal inhibitor partially inhibited MC mediated bone marrow lymphoplasmacytic proliferation and/or tumor colony formation.

Nonomura et al[62] demonstrated in prostate cancer that MC counts were higher around cancer foci in patients with higher Gleason scores than in those with low Gleason scores. The MC number correlated with clinical stage and multivariate analysis revealed that MC count was a significant prognostic factor. By contrast, high intratumoral MC density was associated with favourable tumor characteristics and a good prognosis in prostate cancer.[63] These contradictory findings are consistent with a role for MCs in the immunological host-defense reaction to prostate cancer.

Gomes et al[64] detected an increased MC density in actinic cheilitis and in squamous cell carcinoma of the lip compared to normal oral mucosa, suggesting a role for MCs in the development and progression of these lesions. Similar findings were reported by Lago Costa et al.[65] In another study, cyclooxygenase (COX)-2 over-expression, tryptase-positive MCs and proteinase-activated receptor (PAR)-2 were investigated in normal lip biopsies and in lip biopsies with actinic cheilitis.[65] MC-derived tryptase was found to induce COX-2 expression by the cleavage of PAR-2. Increased epithelial co-expression of COX-2 and PAR-2, as well as an increase in the subepithelial density of tryptase-positive MCs were found in actinic cheilitis compared to normal lip. COX-2 overexpression was found to be a significant predictor of actinic cheilitis and to be correlated with both tryptase-positive MCs and PAR-2 expression. The authors concluded that tryptase may contribute to COX-2 up-regulation by epithelial PAR-2 activation during early lip carcinogenesis.[66] However, the number of tryptase- and KIT-positive MCs was found to be decreased in oral squamous cell carcinoma biopsies by Oliveira-Neto et al.[67]

Aaltomaa et al[68] found a positive correlation between survival and increased MC number in a study of 187 breast cancer biopsies. Dabiri et al[69] analyzed the correlation between MC number in breast cancer and patients' prognosis in a study of 438 patients. They found a strong correlation between the presence of MCs and a favourable prognosis. Perivascular tumor-associated MCs in mammary adenocarcinoma could secrete several cytokines and proteolytic enzymes that could be detrimental to the tumor cells. For instance, IL-4, which binds to IL-4 receptors expressed by human breast carcinoma cells, could lead to apoptosis in breast cancer.[70] However, the histamine content of human breast cancer tissue is much higher than adjacent normal tissue and could act as a local immunosuppressant.[71] Moreover, the mean level of serum tryptase in women with breast cancer is three-times higher than in healthy women.[72] Kankkunen et al[73] similarly observed that the significant increase in MC counts in breast carcinoma versus benign lesions is due to tryptase-containing MCs. In benign lesions, the number of MCs exhibiting tryptase activity was similar to that of chymase containing MCs. Malignant tumors, however, had 2-3 times more tryptase-containing, than chymase-containing, MCs and the tryptase

activity was significantly higher than in benign lesions. Moreover, in malignant lesions, tryptase-containing MCs were concentrated at the tumor edge, whereas chymase-containing MCs were not increased in this area. Breast cancer patients with metastases in the axillary nodes revealed greater numbers of MCs in all nodes examined, compared with patients without metastasis.[74] Della Rovere et al,[75] however, argued that MCs may play a protective role against breast cancer cells. They found that there was a highly significant increase of MCs in infiltrating ductal breast cancers having high hormone receptor content with respect to both hypo-hormonal cancers in the same location and to controls. In another study, stromal MCs were found to correlate to low grade invasive breast carcinoma and estrogen receptor positivity.[76]

Welsh et al[77] analyzed the presence of MCs in the tumor stroma of 175 patients with surgical resected non small cell lung carcinoma. They demonstrated that both macrophage and MC infiltration of the tumor islets was associated with a marked increase in 5-year survival, independently of other favourable prognostic factors including stage. Chan et al[78] studied samples of ovarian cancer from 44 patients and demonstrated that patients with tumors with higher microvascular density had a higher mean survival compared to those with tumors with low MC density or low microvessel density. Cinel et al[79] showed a significant correlation between high MC density and the presence of myometrial invasion in endometrial carcinomas. An increase in the number of tumor infiltrating tryptase-positive MC after IL-2 preoperative induction therapy predicted an improved clinical outcome in patients with malignant pleural mesothelioma. This highlights the critical role of the local inflammatory response in mesothelioma cancer progression.[80]

ANGIOGENESIS FACTORS STORED IN MAST CELLS

MCs synthesize and store large amounts of molecular compounds exerting remarkable activity upon different aspects of angiogenesis. Heparin stimulates endothelial cell proliferation and migration in vitro.[81,82] In vivo, however, it has been reported to stimulate,[83-85] inhibit [85-87] or have no effect[88,89] on angiogenesis. These differences seem to be related to the molecular size and degree of sulphation of the heparin employed. Heparin acts as a soluble form of the low-affinity FGF-2 receptor,[90] displacing FGF-2 in the biologically active form and allowing FGF-2 rapid interaction with endothelial cells.[91]

Histamine has an angiogenic effect through both H1 and H2 receptors.[92] It may also increase the permeability of newly formed microvessels during tumor angiogenesis and hence increase the leakage of plasma proteins and deposition of fibrin. Degradation products of fibrin are angiogenic in vivo.[93]

MC synthesize and store large amounts of MMP-2, MMP-9 and serine-proteinases of two subclasses: tryptase and chymase.[94-96] Given the ability of MMP-2 and MMP-9 to degrade Type IV, V, VII and X collagens and fibronectin,[97] the major components of the interstitial stroma and subendothelial basement membrane, these facts suggest that MCs may contribute to the in situ progression of invasive and metastatic solid tumors.[97] Tryptase and chymase are involved in angiogenesis after their release from activated MC granules. Their proteolytic activities degrade ECM components, release matrix-associated growth factors,[98] and act indirectly by activating latent MMP[99] and plasminogen activators.[32] Blair et al[100] have demonstrated the angiogenic potential of tryptase in vitro and its important role in neovascularization. Muramatsu et al[101,102] used the hamster sponge-implant model to show that angiogenesis is induced by angiotensin

II and inhibited by chymase inhibitors. These observations suggest that MC-derived chymase is an important mediator of MC-dependent angiogenesis.

MCs release polypeptide growth factors including FGF-2, VEGF, TNF-α, TGF-β and IL-8. These cytokines are involved in normal, as well as tumor-associated angiogenesis. The spectrum of cytokines expressed appears to vary depending on the maturity state of the MC and the tissue of residence. Qu et al[103] demonstrated that FGF-2 is localized in the cytoplasmic and extruded granules of MCs in several human tissues. Grutzkan et al[104] demonstrated the expression of VEGF in the human MC line HMC-1 and in human skin MCs. Nerve growth factor (NGF), also contained in MC secretory granules, induces endothelial cell proliferation in vitro and angiogenesis in vivo in the chick embryo chorioallantoic membrane (CAM) assay.[105,106]

MAST CELLS IN EXPERIMENTAL TUMOR ANGIOGENESIS

Kessler et al[107] demonstrated that tumor angiogenesis factor (TAF) elicited a vasoproliferative response when placed upon the CAM of the chick embryo. The action of TAF might depend upon recruitment of MCs. Poole and Zetter[108] demonstrated that rat peritoneal MCs migrate in response to conditioned medium from several tumor cell lines. The active chemoattractant(s) in this conditioned medium appeared to be peptide(s) with a molecular weight of 300-1,000. They proposed that the chemoattraction of MCs by tumor-derived peptides may be an important early event in tumor neovascularization. Starkey et al[109] investigated the role of host MCs in tumor-associated angiogenesis by comparing the angiogenic response of genetically MC-deficient W/W^v mice and MC-sufficient $^{+/+}$ litter mates to subcutaneously growing B16-BL6 tumors. The response was slower and initially less intense in W/W^v mice. Fewer W/W^v mice compared to $^{+/+}$ mice developed spontaneous lung metastases. Bone marrow repair of the MC deficiency restored the incidence of haematogenous metastases to approach that of $^{+/+}$ mice. These results demonstrate a role for MCs in vivo during tumor angiogenesis and also suggest a role for host MCs in hematogeneous metastases.

We have demonstrated that isolated MCs or their secretory granules, but not degranulated MC, induce an angiogenic response in the CAM assay.[110] Addition of anti-FGF-2 or anti-VEGF antibodies reduced the angiogenic response of both MCs and their secretory granules by 50% and 30% respectively. These data provide evidence that the angiogenic properties of MCs depend on the angiogenic molecules contained in their secretory granules and indicate that FGF-2 and VEGF are the angiogenic cytokines primarily and perhaps synergistically, responsible for this vasoproliferative activity.

MAST CELLS IN HUMAN TUMOR ANGIOGENESIS

In the uterine cervix, tryptase-positive MCs increase in number and vascularization increases in a linear fashion from dysplasia to invasive cancer.[111] We have demonstrated that angiogenesis in human endometrial carcinoma is highly correlated with MC tryptase-positive cell counts and that these parameters increase with tumor progression.[112] An association of VEGF and MCs with angiogenesis has been demonstrated in laryngeal carcinoma,[113] in non small cell lung carcinoma where most intratumor MCs express VEGF,[114-116] and in melanoma where MCs express both VEGF[117] and FGF-2.[118] Lastly, a

prognostic significance has been attributed to MC and microvascular density in squamous cell cancer of the oesophagus,[119] squamous cell carcinoma of the oral cavity[52] and in melanoma.[51] Imada et al[114] studied 85 cases of Stage I non small cell lung carcinoma and demonstrated a higher number of tryptase-positive MCs compared to cases of squamous cell carcinoma. A high correlation was observed between intratumor MC counts and microvessel counts; and double immuno-staining showed that most intratumor MCs expressed VEGF.

In melanoma, MC accumulation around the margin of tumors has been observed to peak just as the tumor acquires the angiogenic phenotype and peri-tumor MC counts correlated strongly with microvascular density, melanoma progression and prognosis.[51,117,118] Furthermore, in melanoma, MCs were closer to each other and to vessels.[120] This close association between MCs and the endothelium may indicate that MCs are involved in the maintenance and long lasting functional integrity of the endothelium.

Vascular density has been shown to be associated with a worse prognosis in Merkel cell carcinomas.[121] Other factors associated with a worse outcome included tumor size, the presence of lymphovascular invasion and tumor MC count. The prognostic significance of microvessel density and MC density correlated with the survival of Thai patients with primary colorectal cancer.[122] A significant positive correlation was found between microvessel density and MC density in the areas of highest vascular density. Patients with tumors of low microvascular density and low MC counts had significantly longer survival rates than those with hypervascular and high MC counts. Significant positive correlations between the microvessel density and MC counts in primary colorectal cancer were found also by Gulubova and Vlaykova.[123] Patients with hypovascular tumor tissues had significantly longer survival than those with hypervascular tumor biopsies. An analogous significant correlation was observed for tryptase-positive MC density: patients with low MC density had significantly better prognosis compared to those with high MC density. We have demonstrated that angiogenesis increases in parallel to the number of tryptase-positive MCs in lymph nodes from patients with breast cancer and that their values are significantly higher in lymph nodes with micrometastases as compared with those without metastasis.[124] In addition, significant correlations have been found between the number of tryptase-positive MCs, the area occupied by MCs positive for tryptase, the microvascular density and the endothelial area in early breast cancer patients.[125]

Angiogenesis in benign lymphadenopathies and B-cell non-Hodgkin's lymphomas (B-NHL), measured as microvessels counts, was correlated with the total and MC tryptase-positive counts. Both increased in step with the increase in malignancy grades.[126,127] In NHL, the cellular expression of VEGF and FGF-2, as well as MC and vessel counts, has been assessed.[58] The number of MCs were greater in T-cell lymphomas than in B-cell lymphomas; and in all NHL a significant correlation was found between vessel count and number of MCs in healthy lung and between vessel count and number of VEGF-expressing cells. Double fluorescence staining for VEGF mRNA and MC tryptase revealed that MCs expressed VEGF mRNA. These data suggest that MCs intervene in angiogenesis in these lesions by expressing VEGF. Glimelius et al[128] evaluated the relationship between the number of MCs and the microvessel count in tissue samples from NHL-involved lymph nodes by immunohistochemistry and did not find any correlation between a high microvessel count and the number of MCs.

Angiogenesis represents an essential step of disease progression in other haematological malignancies. In Waldenström's macroglobulinemia (WM), the bone marrow microvessel density is increased in 30-40% of patients, but this seems to have no impact on survival.[129]

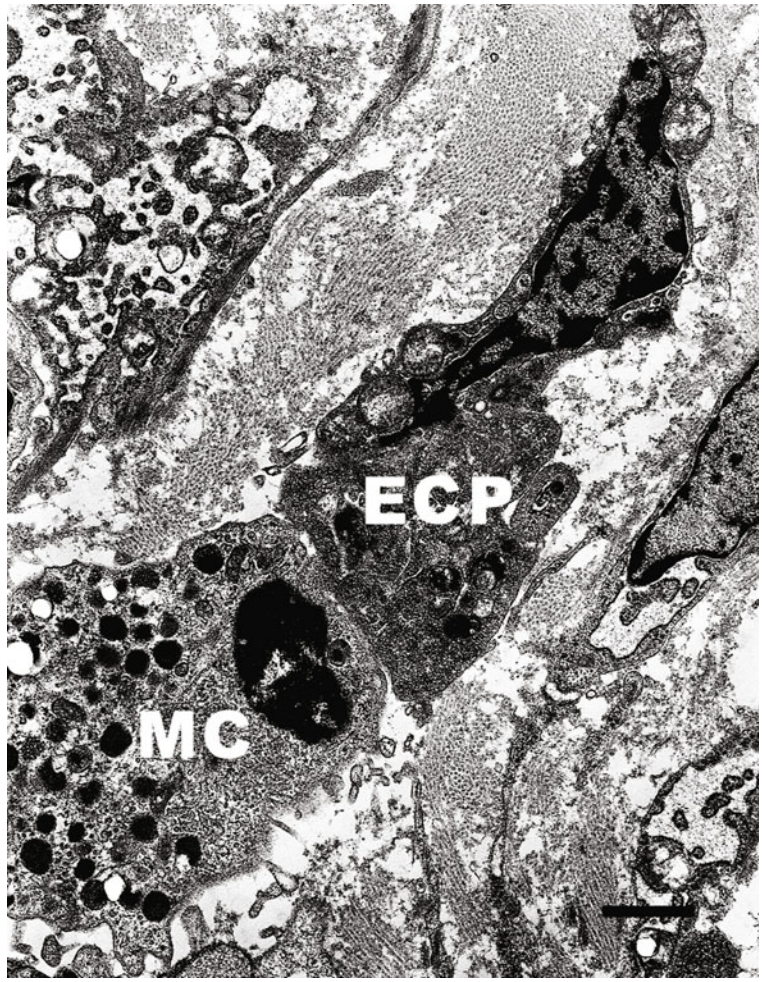

Figure 2. Electron micrograph of a human MC from the lymph node in a patient with B-cell non-Hodgkin's lymphoma. This MC is embedded in an area of intense angiogenesis and establishes a close contact with an endothelial cell precursor (ECP). Bar = 1 μm.

Angiogenic cytokines produced by MCs, such as angiogenin, VEGF and FGF-2, are increased in the serum of WM or IgM- monoclonal gammopathies of undetermined significance (MGUS) patients. Bone marrow angiogenesis, evaluated as microvessel area and MC counts are highly correlated in patients with inactive and active multiple myeloma (MM) and in those with MGUS. Both parameters increase simultaneously in active MM.[130] In both B-NHL and MM, MCs rest near or around blood or lymphatic capillaries (Fig. 2). Their ultrastructural picture includes piecemeal degranulation of their secretory granules, unlike the IgE-mediated massive degranulation that occurs during immediate hypersensitivity reactions.[126,130-132] This morphology is typical of the slow degranulation that takes place in delayed hypersensitivity reactions and chronic

inflammation.[133] It may reflect slow but progressive release of angiogenic factors which favor chronic and progressive stimulation of MC degranulation.[134]

Bone marrow samples of patients with myelodysplastic syndromes display a high correlation between microvessel counts and both total and tryptase-positive MCs. In addition, both parameters increase simultaneously with tumor progression.[135] There is also a correlation between the extent of angiogenesis and the number of tryptase-positive MCs in patients with early B-cell chronic lymphocytic leukaemia and tryptase-positive MCs predict the clinical outcome.[136,137]

DRUGS AFFECTING MAST CELL NUMBER

Preliminary studies using anti-KIT antibodies,[14] anti-TNF-α antibodies[6] or the MC stabilizer disodium cromoglycate (cromolyn)[7] in mouse models, have demonstrated promising results even if administered after the initiation of tumor development. Treatment of mice bearing mammary adenocarinoma and pancreatic cancer with cromolyn led to clotting of blood vessels, hypoxia and tumor cell apoptosis.[7,72] Unfortunately, cromolyn is a weak inhibitor of human MC secretion and is poorly adsorbed, so it is unlikely to be effective in treating cancer in humans.

The first tyrosine kinase inhibitor introduced into the clinic, STI571 (imatinib mesylate, Gleevec), has inhibitory activity against the signalling cascade activated through KIT (CD117).[138] This inhibitory activity is the basis of the use of this drug against gastrointestinal stromal tumors (GIST), most of which harbour a KIT mutation.[139] STI571 is also in use for some varieties of mastocytosis, although some KIT activating mutations involved in mastocytosis are resistant to its inhibitory activity.[140] Ranitidine, an H2 receptor antagonist, when used as adjunctive therapy, prolonged survival of colorectal cancer patients.[141] Another H2 receptor antagonist, famotidine, given pre-operatively, enhanced tumor infiltrating lymphocytes and increased metastatic lymph node reactive changes in breast cancer in humans.[142] Molica et al[143] demonstrated that the reduction in the extent of bone marrow angiogenesis observed after sequential therapy with low doses of subcutaneous alentzumab after a clinical response to fludarabile induction therapy, was associated to a reduction in the number of MCs .

Dietary supplementation of silymarin, a naturally derived polyphenolic antioxidant, exerts a beneficial role in N-nitrosodiethylamine (NDEA)-induced liver cancer in Wistar albino male rats.[143] NDEA administered rats showed increased MC density, along with upregulated expression of MMP-2 and MMP-9. Silymarin treatment inhibited the increase in MC density and downregulated the expression of MMP-2 and MMP-9 as revealed by Western blotting and immunocytochemistry. Thus, silymarin may exert beneficial effects on liver carcinogenesis by attenuating the recruitment of MCs and thereby decreasing the expressions of MMP-2 and MMP-9.[144]

CONCLUSION

MCs are found in multiple tissues and their presence in association with human cancer has been established for many years. However, multiple reports have described both a positive and a negative correlation between MC number and prognosis in various human tumor types (Fig. 3). These discrepancies are also observed in experimental studies, and

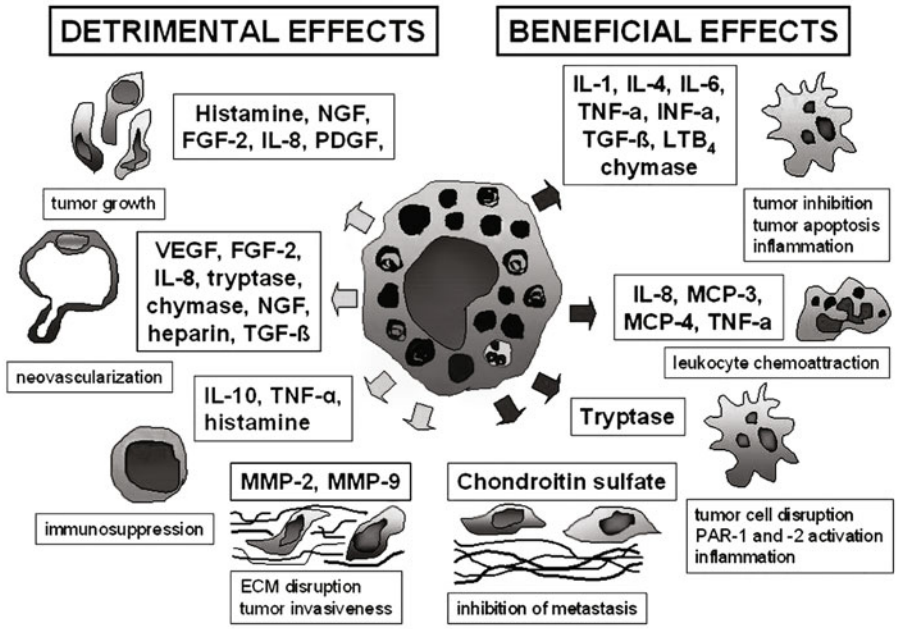

Figure 3. The dual role of MCs in tumor fate. MCs may release cytokines and growth factors in the tumor stroma, such as FGF-2, NGF, PDGF, IL-10 and IL-8, with detrimental effects to the host by stimulating tumor cell expansion. MCs are a major source of histamine, which can induce tumor cell proliferation through H1 receptors, while suppressing the immune system through H2 receptors. In addition, MCs synthesize and store angiogenic factors as well as matrix metalloproteinases, which promote tumor vascularization and tumor invasiveness, respectively. MCs may also generate immunosuppression by releasing IL-10, histamine and TNF-α. By contrast, MCs may promote inhibition of tumor cell growth, tumor cell apoptosis and inflammation by releasing cytokines such as IL-1, IL-4, IL-6 and TNF-α. TNF-α, in particular, is effective in leukocyte chemoattraction. Chondroitin sulphate may inhibit tumor cell diffusion; and tryptase causes both tumor cell disruption and inflammation through activation of protease-activated receptors (PAR-1 and -2). Abbreviations: FGF-2, fibroblast growth factor-2; NGF, nerve growth factor; PDGF, platelet derived growth factor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; INF-α, interferon-α; LTB_4, leukotriene B4; MCP-3, MCP-4, mast cell protease-3, -4; MMP-2, MMP-9, matrix metalloproteinase-2, -9; VEGF, vascular endothelial growth factor; IL-1, IL-2, IL-6, IL-8, IL-10, interleukin-1, -2, -6, -8, -10.

could be due to differences in the mouse model used and the stage of carcinogenesis investigated.

MCs are recruited in tumor development and play a key role in both angiogenesis and tissue remodelling by promoting both tumor initiation and growth. In addition, as tumor growth progresses, MCs recruit immune cells; or alternatively suppress antitumoral responses. Reports addressing MCs specifically as mediators in tumor immunosuppression come from Huang and coworkers.[13] They mobilize MCs to infiltrate tumors by means of SCF. Tumor-derived SCF dependent MC infiltration and activation resulted in remodelling of the tumor microenvironment and tumor growth.

MCs could be a new target for the adjuvant treatment of tumors, through the selective inhibition of angiogenesis, tissue remodelling and tumor-promoting molecules, permitting the secretion of cytotoxic cytokines and preventing MC-mediated immune suppression. Moreover, some of the new targeted anticancer therapies have pronounced effects on

MCs; in fact, it may be that some of their antitumor effects are closely related to their effect on MCs.

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