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ADVANCES IN PHARMACOLOGY
Immunopharmacology

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PREFACE

Let me begin by stating that what follows below is a highly personal view of what has taken place in the general field of immunology and reflects what I have observed over the last 40 years or so of my own work in the areas traditionally called variously immunology, cellular immunology, molecular immunology, immunopharmacology and inflammation. As we shall see, this is also reflected in the authors and subjects that appear in this volume.

It has now been more than thirty years since the first International Congress of Immunopharmacology was held in Brighton, England (1980). Many of us who attended the Congress and gave talks there did not necessarily consider that our research would fall under the rubric of 'immunopharmacology' as most of us considered ourselves simply immunologists or pharmacologists or physician-scientists interested in autoimmune diseases and inflammation. What crystallized for me at that meeting was a notion, which had been knocking around for some time, namely that the use of pharmacologically active agents such as beta agonists and antagonists, cyclooxygenase inhibitors, calcium channel blockers and phosphodiesterase inhibitors could be remarkably useful as probes to understand many of the aspects of immune cell activation and regulation. Thus, the simple definition of immunopharmacology-the study of the effects of pharmacologically active agents on the immune system-came to mean something much more profound for the field of immunology. Subsequent work has proven to me the veracity of this sentiment. As researchers in academia and in Pharma and Biotech companies began to develop a wide array of drugs that influenced the function of immune cells, more and more papers began to appear that made use of these drugs to understand the details of cell signaling pathways in the immune system, how different naturally produced small molecules changed the functioning of the immune system. These results linked multiple tissue and organ systems together as it became clear that substances originally thought of as active in the lung, kidney or brain also have very discreet effects on all types of immune cells. This evolution of the immunopharmacology field to encompass studies on the details of immune cell function and regulation has blurred the original distinctions that researchers viewed as the differences between traditional cellular immunology and immunopharmacology. Moreover, as the number of researchers grew and ever more intensive studies of intracellular signaling pathways in immune cells were reported, new compounds were discovered that supplied additional reagents for probing immune system function. This had the added effect of blurring

the old lines between typical inflammation focused research, immunopharmacology and classical cellular immunology such that those of us who work in these fields tend not to view the distinctions as particularly relevant.

With the rise of 'omics' beginning in the 1990s, which replaced older terms (e.g. genetics became genomics, protein biochemistry became proteomics, etc.), we have arrived at the point where the old academic categories are increasingly giving way to university departments that emphasize crossfertilization of disciplines making specific distinctions even more problematic. Moreover, the tools of the modern molecular biologist, namely the ability to genetically manipulate common laboratory species, like the mouse, have led us to the point where we can test hypotheses about the role of specific proteins in cell function that is quite reflective of pharmacological approaches.

Thus we arrive at the contents of the present volume. As one can see from the author list, they come from a variety of institutions and academic departments. To me what links them together is the use of pharmacologically active compounds to study the functioning of immune cells as well as a style of thinking about research problems that blends together a variety of disciplines, all of which are brought to bear on testing various hypotheses about how the immune system really functions and moreover, how one might apply this knowledge to the immune related diseases that afflict mankind.

For example Altman and collaborators have hypothesized a role for PKC theta in regulating TH2 and TH17 cell function and in this volume they summarize their work that suggests that it is a highly interesting target for pharmacologic intervention in a range of allergic and autoimmune diseases.

Arron and colleagues tackle one of the more historically traditional areas for immunopharmacology, namely allergic disease and asthma, a field that supplied some of the early reagents used to probe not only the action of cells involved in allergic disease but also T-cells that were not originally included as targets in these diseases. The approach is based on the use of human studies and introduces entire new families of pharmacologically active substances, the cytokines and alarmins, that can, at the very least, supplement the old line therapies of corticosteroids and beta-adrenergic agonists.

Hardwick and his group demonstrate one of the other benefits of crossfertilization of disciplines, namely that diseases not traditionally linked to the immune system such as insulin resistance, dyslipidemia and hypertriglyceridemia may, in fact, have links there nonetheless (leading many an immunologist to say only half jokingly, that "all diseases have their basis in the immune system"). Here again, the authors focus on one of the more traditional areas that were once thought of as the province of immunopharmacology, the eicosinoids, and bring new insights into the disease process and the functioning of the immune system.

One of the oldest areas of study in all of immunology is the study of bacterial lipopolysaccarides. Bowen, et al., in Kolb and Mitchell's laboratories give us an update on this fascinating field that has led to a remarkable number of insights as to how immune cells function and which ones have proven to be highly potent pharmacologically active agents in and of themselves. It should be remembered that these agents are highly pathogenic when produced by bacteria during infection. Yet they may be modified in such a way so as to be highly useful adjuvants and stimulants to the immune system. In addition, this paper introduces one of the most fascinating of the recently discovered receptors on immune cells, the toll-like receptors (TLRs) that are now the targets of intense investigation world-wide.

Among the cytokine families that have achieved a unique place in the pantheon of immune modulators, the highly complex TNF superfamily stands almost alone in its effects on modern drug discovery. The discovery of the role of TNF in autoimmune disease has been the subject of numerous reviews and drugs targeting the TNF receptor(s) or TNF itself have resulted in a multibillion dollar drug franchise for several biotechnology and pharmaceutical companies. The work of Ware and his colleagues has opened entirely new chapters in this saga by probing more deeply the biology and pharmacology of this complex system of receptors and ligands including the lymphotoxins and other members of this group (LIGHT). Their work has led to new observations about how members of this family affect development and treatment of autoimmune diseases while underscoring the newer therapeutic opportunities presented as we gain more insight into this fascinating family.

Finally, Ziegler and his colleagues introduce us to the lymphopoetins that turn out to have effects far beyond what was originally thought to be the case. Here we see the connectivity between the various components of the immune system that use a single protein for many more purposes than initially imagined. Thus this protein can affect a wide variety of disorders including autoimmunity but also cancer. This point illustrates the theme of this volume, namely that the study of the immunopharmacology of the immune system has far reaching consequences that span the entire range of human disease.

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CHAPTER ONE

Redefining Approaches to Asthma: Developing Targeted Biologic Therapies

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Abstract

Asthma is a chronic respiratory disorder canonically associated with type 2 airway inflammation as characterized by elevated levels of eosinophils, immunoglobulin E, and cytokines including interleukin (IL) 4, IL5, IL9, and IL13 and tumor necrosis factor (TNF) α. However, mounting evidence has shown that considerable heterogeneity exists in human asthma in terms of the nature and intensity of airway inflammation. While many asthma patients achieve acceptable control of symptoms with standard-of-care therapies such as β_2-adrenergic agonists and inhaled corticosteroids, a minority remains symptomatic despite maximal standard-of-care therapy and constitutes a significant unmet medical need. A growing number of investigational therapeutics under clinical development for asthma are biologic therapies that specifically target mediators of type 2 airway inflammation. In this chapter, we consider the biological functions of therapeutic targets in asthma and data from clinical trials of biologic agents directed against these targets. We discuss recent clinical trial results in terms of four key components of drug development: target selection, molecule selection, outcome selection, and patient selection, with particular attention paid to the emerging role of biomarkers in clinical development for asthma.

1. INTRODUCTION

The ultimate objective of drug development is to deliver clinical benefit to patients with unmet medical needs. To achieve this objective successfully, it is necessary to appreciate the relationships between the molecular targets of drug candidates and objective clinical outcome measures. To link targets to end points, we must gain a more sophisticated understanding of how targets and end points are, in turn, related to the pathophysiology of disease in human subjects. In this chapter, we explore the current understanding of asthma with respect to inflammatory pathways, clinical manifestations, heterogeneity, clinical trial design, and the use of biomarkers to facilitate drug development for biologic therapies. These considerations are assessed in the context of the conceptual relationships between targets, pathophysiology, and end points, depicted schematically in Fig. 1.1.

Drug development is an enterprise characterized predominantly by failure punctuated by occasional successes, rather than the other way around. Recent analyses have shown that only about 12% of therapeutic candidates that enter phase 1 trials successfully end up as marketed products, and this high failure rate contributes substantially to the high costs of drug development (Munos, 2009). While there are many reasons why a drug candidate might fail, recent efforts to develop targeted biologic therapies for asthma help to illustrate how emerging approaches to clinical development for a heterogeneous chronic disease can help to mitigate risk. Four important

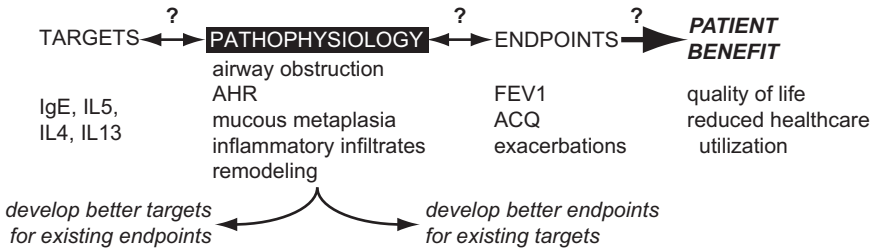

Figure 1.1 ***Asthma pathophysiology links therapeutic targets and clinical end points.*** How therapeutic targets contribute to human asthma pathophysiology and how specific domains of pathophysiology relate to clinical end points are incompletely understood. To improve the drug development process, a better understanding of these relationships will be necessary so that target and end point selection can be optimized to maximize benefit to patients.

reasons why a drug candidate may fail are (1) wrong target—the hypothesized target or pathway is not a critical node in disease pathogenesis and/or unacceptable safety risks are associated with the target; (2) wrong molecule—the therapeutic candidate has insufficient affinity or avidity, has off-target effects, lacks adequate pharmacokinetic (PK) properties to achieve necessary exposures, and/or is inadequately dosed; (3) wrong outcomes—the clinical outcome measure assessed in interventional trials is inappropriate relative to the biology of the targeted pathway and/or the population of patients studied; and (4) wrong patients—patients in the trials are not stratified appropriately to account for molecular, pathophysiological, or clinical heterogeneity of the disease and/or the trial is statistically underpowered to demonstrate benefit in the subset of patients most likely to benefit. Recent clinical trials of targeted biologic therapies in asthma help to illustrate the importance of proper target, molecule, outcome, and patient selection.

2. HUMAN ASTHMA BIOLOGY

2.1. Clinical Features

Bronchial asthma is a chronic respiratory disorder characterized by episodic, reversible obstruction of the airways. The following three clinical and physiologic determinants are necessary and sufficient to define asthma in adults: (1) airway obstruction as assessed by decreased predicted forced expiratory volume in 1 second (FEV1) without loss of predicted vital capacity, (2) reversibility of obstruction to β_2-adrenergic agonists (e.g. albuterol) or systemic corticosteroids, and (3) airway hyperreactivity (AHR) as defined by an acute decrease in FEV1 in response to a provocative agent

(e.g. methacholine, histamine, or mannitol). Associated symptoms include wheezing, shortness of breath, cough, night-time awakenings, and activity limitations as a result of these symptoms (Bateman et al., 2008). Acute worsening of asthma symptoms, or exacerbations, may result in hospitalization and/or systemic steroid treatment and are responsible for a significant proportion of asthma-related morbidity and health care utilization (Jackson et al., 2011). Despite intensive research, the etiology of airway dysfunction in asthma is complex and poorly understood. Consensus definitions of asthma typically include the term "inflammation" (Bateman et al., 2008). Given mounting evidence (discussed in detail below), a more nuanced view is that inflammation, depending on how it is defined, may not always be an essential feature of asthma. However, inflammation is commonly associated with the development and clinical manifestations of asthma, particularly exacerbations, and many new asthma therapies target different aspects of airway inflammation.

Asthma is commonly associated with allergy and is hypothesized to be part of an "atopic march" from eczematous skin inflammation, leading to sensitization to aeroallergens and allergic rhinitis, ultimately progressing to bronchial asthma (Zheng et al., 2011). Recent epidemiologic evidence strongly implicates viral respiratory infections in eczematous children as potentially predisposing to the subsequent development of asthma (Jackson et al., 2012). However, the precise interplay and sequence of events involving allergic sensitization, viral infection, and asthma are not completely understood, and this allergic diathesis does not appear to be present in many patients with asthma, particularly those with adult-onset disease (Haldar et al., 2008; Hashimoto & Bel, 2012; Moore et al., 2010). Furthermore, the relative contribution of ongoing inflammation to disease manifestations of patients with established asthma is not clear. The majority of existing and experimental treatment approaches to asthma focus on treating established asthma rather than preventing incident asthma. Thus, characterizing the active pathways contributing to symptoms in patients with established disease has been the most common approach to identifying targets and developing new asthma therapies.

2.2. Current Standard of Care

Guidelines for standard-of-care asthma therapy are empirical and are based on clinical presentation rather than the underlying biology of disease. The current pharmacopoeia of approved asthma therapies is composed of inhaled short-acting β_2-adrenergic agonists (SABAs), inhaled long-acting

β_2-adrenergic agonists (LABAs), inhaled corticosteroids (ICSs), oral corticosteroids (OCSs), oral leukotriene receptor antagonists (LTAs), theophylline, anticholinergic agents, and injectable omalizumab (humanized monoclonal antibody (mAb) to IgE). Bronchial thermoplasty (BT) is a bronchoscopic procedure that has recently been approved for refractory asthma that ablates bronchial smooth muscle using the focal application of heat (Thomson et al., 2012; Wahidi & Kraft, 2012). Typically, asthma treatment guidelines recommend "step-up" or "step-down" therapy according to the level of symptom control achieved at a given level of therapy (Bateman et al., 2008; BTS, 2008; EPR-3, 2007). These "steps" start with SABAs as needed for the mildest patients. Patients not achieving adequate control at that step remain *pro re nata* on SABAs with the introduction of increasing doses of daily ICS treatment and then the addition of other controller medications such as LABAs, LTAs, theophylline, anticholinergics, and ultimately OCS until adequate symptom control is achieved, with the goal of eventually "stepping down" to the minimal level of treatment needed to maintain control. For some asthma patients in whom there is poor control despite maximal doses of ICS ± LABA ± OCS, omalizumab (in patients with allergic asthma) or BT may be considered. While a majority of asthma patients achieve adequate control on some combination of inhaled therapies, a substantial number of patients do not achieve controlled despite maximal standard-of-care therapy, consume a disproportionately high amount of health care expenditures, and represent the major unmet medical need in asthma (Braman, 2006). Asthma severity is defined in terms of the level of treatment required to achieve symptom control, with "mild" asthmatics generally achieving adequate control with SABAs, "moderate" asthmatics requiring but achieving adequate control with ICSs, and "severe" asthmatics not achieving control despite maximal ICS ± LABA ± OCS. In recent years, biologic antiinflammatory therapies have been under development for this "severe" segment of the asthma population (Catley et al., 2011; Holgate, 2012).

2.3. Airway Inflammation in Asthma

2.3.1. Inflammatory Cytokines

Airway inflammation associated with asthma is generally associated with the so-called Th2 cytokines, typically produced by activated CD4+ T cells polarized in the presence of interleukin (IL) 4. Cytokines produced by Th2 cells canonically comprise IL4, IL5, and IL13, but many other cytokines from a range of cellular sources, including IL9, IL17, IL25, IL33, thymic stromal

lymphopoietin (TSLP), and tumor necrosis factor (TNF) α, among others, have been implicated in asthmatic airway inflammation (Barnes, 2008; Catley et al., 2011; Holgate, 2012). Further complicating matters, classical Th2 cells are neither the sole nor even perhaps the dominant source of these cytokines in vivo in humans with established asthma. For example, IL13 is expressed by nonclassical helper T cells including CD4+ cells that produce both Th2 and Th17 cytokines (Cosmi et al., 2010; Wang et al., 2010) and CD8+ T cells (Gelfand & Dakhama, 2006) as well as by non-T cells including mast cells, eosinophils (Berry et al., 2004), natural killer T cells (Akbari et al., 2003), macrophages (Kim et al., 2008), nuocytes (Barlow & McKenzie, 2011), and type 2 myeloid (T2M) cells (Petersen et al., 2012). Hence, while "Th2" has historically been shorthand nomenclature for this pattern of inflammation, a more appropriate and general term acknowledging cellular sources of cytokines beyond only T cells might be "type 2 inflammation" (Pulendran & Artis, 2012). Figure 1.2 is a schematic diagram of some of the key components of type 2 inflammation in asthma.

Mucosal damage mediated by environmental insults such as allergens, viruses, bacteria, fungi, or pollutants results in the release of IL25, IL33, and TSLP, termed "type 2 alarmins," from the epithelium (Bartemes & Kita, 2012). These cytokines act on innate immune cells including dendritic cells, mast cells, and nuocytes, leading to the activation of adaptive immune cells including T and B lymphocytes. The products of each of these cellular mediators can provide feedback on and amplify the inflammatory cascade, resulting in elevated levels of type 2 effector cytokines, particularly IL5 and IL13, in the bronchial mucosa. Regardless of the cellular sources of these cytokines, they mediate a variety of effects on the airway. IL4 can amplify Th2 polarization of CD4+ T cells and promote activation and isotype switching of B cells to produce IgE. IL5 promotes the differentiation, mobilization, and survival of eosinophils. IL13, like IL4, acts on B cells to promote isotype switching to IgE; it also acts on structural cells of the airway, contributing to mucus-producing goblet cell differentiation from epithelial cells (mucus metaplasia), fibrosis, and expression of eosinophil-attracting chemokines (Barnes, 2008). IL9 is produced by some activated Th2 cells as well as a "Th9" cell lineage that expresses IL9 in the absence of IL4, IL5, and IL13 (Jabeen & Kaplan, 2012). IL9 is thought to play roles in the recruitment and activation of mast cells in the lung (Jones et al., 2009). TNFα is produced by activated cells of the myeloid lineage and multiple lineages of activated T cells including Th2 cells differentiated in the presence of OX40L (Wang et al., 2006). It mediates a wide variety of effects on target cells, including

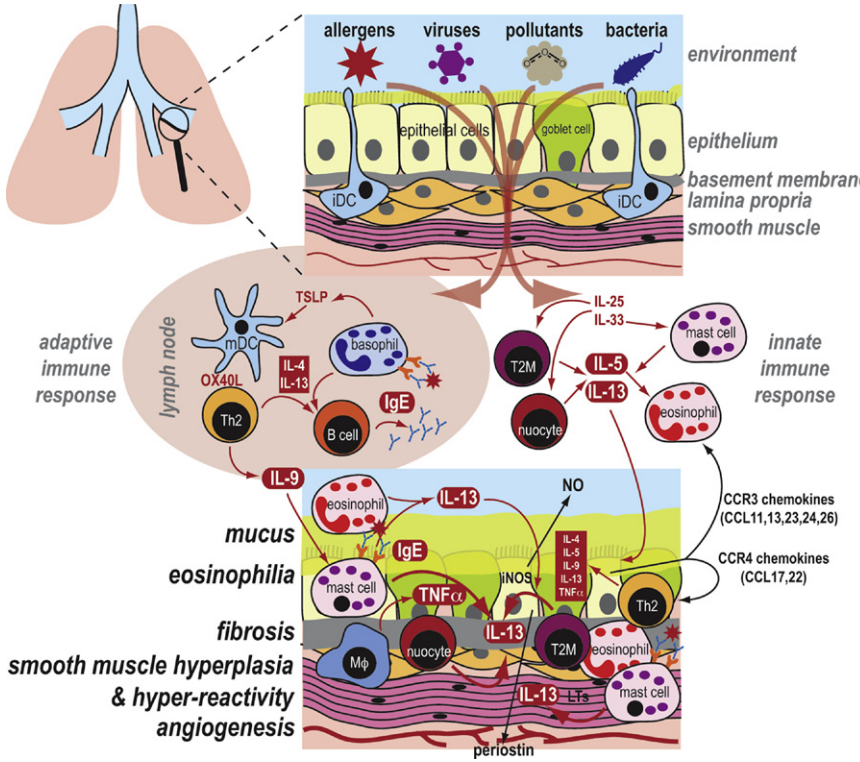

Figure 1.2 ***Mediators of type 2 inflammation in human asthma.*** The bronchial mucosa is exposed to environmental stimuli such as allergens, viruses, pollutants, and bacteria. Epithelial damage leads to the release of "type 2 alarmins" such as TSLP, IL25, and IL33, which activate cells of the innate immune system such as dendritic cells (iDC, immature dendritic cell; mDC, mature dendritic cell), basophils, mast cells, type 2 myeloid (T2M) cells, nuocytes, eosinophils, and macrophages (MΦ). DCs direct adaptive immune responses, promoting differentiation of Th2 cells and isotype switching of B cells to produce IgE. Type 2 cytokines including IL4, IL5, IL9, and IL13, as well as other inflammatory mediators such as TNFα and leukotrienes (LTs), act on infiltrating leukocytes and resident cells of the airway including epithelial cells, subepithelial fibroblasts, and airway smooth muscle to induce the hallmark pathophysiological changes of asthma including mucus metaplasia, subepithelial fibrosis, airway smooth muscle hyperplasia, airway hyperreactivity, and angiogenesis. Therapeutic targets for biologic agents discussed in this chapter include IgE, IL4, IL5, IL9, IL13, and TNFα. Biomarkers used in clinical studies including CCR3- and CCR4-binding chemokines, exhaled nitric oxide, and periostin are indicated. (For color version of this figure, the reader is referred to the online version of this book).

the induction of neutrophil chemoattractants. While the precise cellular origins of, and interplay between, each of these effector cytokines remain unclear in human asthma, it is reasonable to interrogate whether inhibiting them may alter airway inflammation sufficiently to provide clinical benefit.

2.3.2. Effects of Corticosteroids on Airway Inflammation

Typically, Th2 cells, eosinophils, and downstream signals emanating from type 2 cytokines are sensitive to the effects of corticosteroids, and for the majority of patients with asthmatic airway inflammation, ICS therapy with or without LABAs and/or LTAs is effective. The unmet medical need in asthma arises in patients who experience no or incomplete benefit from ICS treatment, and this subset of patients has been variously described as patients with "difficult," "refractory," "severe," or "brittle" asthma (Blakey & Wardlaw, 2012; Hashimoto & Bel, 2012). There are several nonmutually exclusive reasons why a given asthma patient may not achieve effective control by ICS therapy: (1) relative genetic insusceptibility to the action of corticosteroids (Tantisira et al., 2011); (2) acquired resistance to steroids, in which a patient who has in the past achieved effective control on ICS gradually becomes less well controlled and/or requires increasing doses of ICS or the addition of OCS to maintain control (Adcock & Barnes, 2008); (3) drivers of disease that are not inherently susceptible to the actions of steroids; and (4) incomplete adherence to the prescribed regimen of steroid treatment (Heaney & Horne, 2012). While it is difficult to account for each of these potential mechanisms of inadequate asthma control despite ICS therapy, many studies have indicated that asthma patients most likely to show clinical benefit from steroid treatment or dose modulation are those with type 2 and/or eosinophilic airway inflammation (Cowan et al., 2010; Green et al., 2002; Jayaram et al., 2006; Woodruff et al., 2009). Given that most of the biologic therapies being developed are targeted at ICS-refractory asthmatics despite the fact that ICSs tend to inhibit the same pathways that these therapies target (i.e. type 2/eosinophilic inflammation), it is vital to understand whether the targeted pathways are indeed active in these poorly controlled patients already on ICS.

2.4. Asthma Heterogeneity

While many asthmatics exhibit characteristic features of type 2 inflammation including elevated IgE levels, sensitivity to aeroallergens, and increased numbers of airway eosinophils, this is not the case in all asthma patients. A growing body of literature has described asthma heterogeneity in terms of the nature and intensity of eosinophilic infiltration in the airways, with strong evidence for variability in airway eosinophilia across the spectrum of asthma severity (Anderson, 2008; Gibeon & Chung, 2012; Hashimoto & Bel, 2012; Hastie et al., 2010; Lotvall et al., 2011; McGrath et al., 2012;

Simpson et al., 2006; Wenzel, 2012; Wenzel et al., 1999; Woodruff et al., 2009). "Mild" asthmatics, not taking ICSs, can be categorized as "eosinophilic" and "noneosinophilic" subsets defined by the percentages of eosinophils in sputum or bronchoalveolar lavage (BAL) fluid or the relative numbers of eosinophils infiltrating the bronchial mucosal tissue in biopsy specimens. Using genome-wide transcriptional analysis of bronchial epithelial brushings in mild-moderate asthmatics, we have defined a "signature" of three genes that can be specifically induced by IL4 or IL13 in vitro: periostin, CLCA1, and serpinB2. This "Th2 signature" was expressed at elevated levels in approximately half of mild to moderate asthmatics, but, on the basis of epithelial expression of these three genes, the other half of asthma patients was indistinguishable from healthy controls, despite having comparable clinical manifestations of disease (i.e. airway obstruction, AHR, and reversibility). The Th2 signature was strongly correlated with the expression levels of IL5 and IL13 in bronchial mucosa, and "Th2-high" asthma patients had elevated eosinophils in BAL fluid and peripheral blood compared to "Th2-low" asthma patients and healthy controls. Importantly, a course of ICS therapy both reduced the activity of type 2 cytokines in the "Th2-high" patients and provided clinical benefit in terms of lung function in the "Th2-high" patients, while the "Th2-low" patients did not show any lung function benefit from ICS (Woodruff et al., 2009). An expansion of this dichotomous epithelial gene signature in concomitantly collected bronchial mucosal tissue to a set of 79 highly coregulated genes that could be summarized as an aggregate score revealed that the intensity of this inflammatory signature varied continuously across asthma patients (Choy et al., 2011) and that the intensity of type 2 inflammation as defined by this signature score ("Th2 sig") correlated continuously with AHR *within asthmatics* as assessed by sensitivity to methacholine. Nevertheless, while the Th2 signature was continuously distributed across asthmatics and healthy controls, there was a substantial discontinuity between asthmatics and nonasthmatics in the study with respect to AHR (Fig. 1.3). Thus inflammation alone does not explain why all the asthma patients in the study had a 20% decrease in FEV1 in response to 8 mg/ml or less of methacholine, while the healthy controls exhibited little or no AHR, suggesting that AHR is an intrinsic feature of asthma that can be modulated by inflammation rather than a direct result of inflammation.

Many other studies have used various means of airway sampling to demonstrate similar heterogeneity across a spectrum of asthma severity, and while the methodology and definitions of "cutoffs" vary in defining

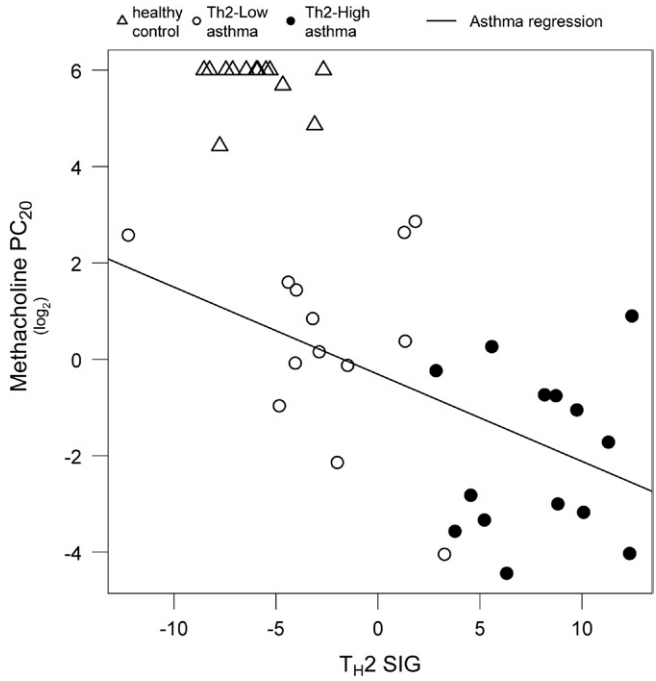

Figure 1.3 ***Disconnection between airway hyperreactivity (AHR) and type 2 inflammation.*** Sensitivity to methacholine (PC_{20}, provocative concentration in milligrams per milliliter needed to produce a 20% decrease in FEV1) is plotted as a function of a bronchial mucosal Th2 gene expression signature (Th2 sig) as described in Choy et al. (2011). Among asthma patients, there is a continuous relationship between methacholine sensitivity and Th2 sig (decreasing PC_{20} denotes increasing sensitivity). However, Th2 sig score between healthy nonasthmatic controls and "Th2-low" asthmatics is overlapping, despite insensitivity to methacholine in the healthy controls. Thus, type 2 inflammation contributes to AHR in asthmatics but does not completely account for it.

phenotypes according to granulocytic infiltration of the airways, the common denominator in these definitions is the relative intensity of airway eosinophilia. In general, a subset of patients with "mild" asthma (defined as controlled on SABAs and not requiring or not taking ICSs) tend to exhibit elevated airway eosinophils and type 2 cytokines, while patients with "moderate" asthma (defined as requiring, but achieving disease control on, ICS therapy) tend not to have substantial airway eosinophilia or expression of type 2 cytokines. However, a subset of patients with "severe" asthma (defined as poorly controlled despite high-dose ICS therapy ± OCS) exhibit reemergence of eosinophilic airway inflammation and elevated levels of type 2 cytokines (Saha et al., 2008). It has been hypothesized that

this steroid-resistant inflammation may be driven more by innate immunity than by adaptive immunity (Anderson, 2008), although this remains to be demonstrated formally. The roles of other granulocytes, particularly neutrophils, are less well defined in asthma, although airway neutrophilia, however it is assessed, appears to be a feature of more severe forms of the disease and may be a cause, a consequence, or both, of relative insensitivity to chronic high-dose ICS exposure (Cowan et al., 2010; Hauber et al., 2003; Saffar et al., 2011; Wenzel et al., 1999). While the precise definitions of asthma subtypes vary, the overlap between "type 2," "Th2-high," and "eosinophilic" asthma is substantial. Thus we will refer to this subtype as "type 2/eosinophilic" asthma for the remainder of this chapter, except in specific cases where precise definitions are contained within references discussed from the literature, where we will use the cited definition.

2.5. Asthma Biomarkers

A National Institutes of Health (NIH) panel defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" (NIH, 2001). Biomarkers have the potential to facilitate drug development for, and clinical management of, asthma on several levels: as predictive, prognostic, pharmacodynamic (PD), and surrogate measures of disease activity.

2.5.1. Types of Biomarkers

Predictive biomarkers provide evidence of the activity of a particular molecular pathway prior to treatment and identify a subpopulation of patients most likely to benefit from a targeted therapy. A given pathway (e.g. type 2/eosinophilic inflammation) may be heterogeneously expressed across a population of asthma patients and the ability to identify clinical benefit from agents targeting that pathway may be compromised if only a subset of patients that cannot otherwise be prospectively identified exhibit benefit. A predictive biomarker that identifies asthma patients most likely to benefit from a targeted therapeutic could help stratify enrollment in clinical trials to more rigorously test the therapeutic hypothesis. Importantly, *to test the therapeutic hypothesis,* it is equally important to demonstrate that patients predicted not to benefit from the intervention actually fail to benefit from the intervention as it is to demonstrate that patients predicted to benefit from the intervention actually do benefit from the intervention, preferably via stratified analyses within clinical trials rather than through historical

comparisons between trials. Ideally, a biomarker to predict benefit from a therapy targeting type 2 inflammation in asthma should have the following characteristics: (1) it should accurately reflect airway inflammation, (2) it should be mechanistically linked to the therapeutic target, (3) it should be readily detectable in patients with asthma, (4) there should be robust, standardized, reproducible assays on widely available platforms.

Prognostic biomarkers can stratify the risk of future disease activity that may be relevant in clinical trial design, e.g. in a trial with severe asthma exacerbations as an outcome measure; a biomarker that is prognostic for the likelihood of future exacerbations might be useful either to stratify enrollment across active and placebo arms or to restrict enrollment to patients most likely to have an exacerbation event during the course of the study so that the statistical power to interpret outcomes is increased. A past history of asthma exacerbations is itself prognostic for future exacerbations (Miller et al., 2007), hence this objectively measurable clinical feature of a given asthma patient meets the definition of a prognostic biomarker.

PD biomarkers should reflect the activity of a particular molecular pathway involved in the disease process and should change in response to a specific therapeutic intervention. Changes in PD biomarkers on treatment indicate whether and to what extent the molecular intervention is affecting its target. PD biomarkers may help to enable appropriate dose selection in a dose-ranging study, enable go/no-go decisions in early clinical studies (where primary outcomes are typically safety related rather than efficacy related), and define the pharmacological activity of a new therapeutic in patients. Significant PD effects in the absence of any clinical benefit may help discriminate between inappropriate target selection and inappropriate dosing as the reason for failure of a trial.

Surrogate biomarkers, as is the case for PD markers, should change in response to treatment but may be distal to the targeted pathway. A key distinction for the purpose of this definition is that surrogate biomarkers are linked directly to downstream manifestations of disease and clinical outcomes, whereas a relationship to disease outcomes may be, but is not necessarily, the case for PD biomarkers. Changes in surrogate biomarkers over the short term may indicate the likely long-term efficacy of continued treatment, e.g. on exacerbation rates.

2.5.2. Biomarkers of Airway Inflammation

Many biomarkers associated with asthmatic airway inflammation have been described, including the cellular content of induced sputum or blood,

exhaled gases, urine metabolites, genetic markers, and soluble proteins in serum or plasma (Szefler et al., 2012). The most commonly described method for directly sampling the airway to assess inflammation is sputum induction. In healthy volunteers, sputum is predominantly composed of macrophages and neutrophils, with eosinophils being rare to nonexistent (Belda et al., 2000). In a subset of asthma patients, a small proportion of sputum cells may be eosinophils. While there is currently no consensus on collection methods or on defined cutoffs for sputum eosinophilia, generally an asthma patient with greater than 2–3% eosinophils in an induced sputum sample is considered "eosinophilic" (Belda et al., 2000; Green et al., 2002; Green et al., 2002; Haldar et al., 2009; Hastie et al., 2010; McGrath et al., 2012; Simpson et al., 2006). Sputum induction, while feasible in specialty pulmonary practices with appropriate laboratory facilities, is time consuming and labor intensive and a suitable sample is not always produced, which, coupled with the lack of standardization, has hindered its widespread adoption.

Although many analytes have been assessed in condensates of exhaled breath, the technology for and implications of exhaled breath condensate biomarkers are poorly understood. However, fractional exhaled nitric oxide (FeNO) is now a fairly well-established approach to assessing airway inflammation (Dweik et al., 2011). NO is produced in the airways by the action of inducible nitric oxide synthase (iNOS), an enzyme induced in bronchial epithelial cells by cytokines such as IL13 (Chibana et al., 2008; Jiang et al., 2009), and can be measured in exhaled breath by point-of-care instruments. Elevated FeNO levels in asthma are indicative of airway eosinophilia but must be interpreted with consideration given to the severity of asthma and concomitant steroid exposure, which can shift FeNO levels downward (Dweik et al., 2011, 2010; Nair et al., 2010; Pavord & Shaw, 2008; Silkoff et al., 2005).

Eosinophil-specific oxidative enzymes can produce metabolites such as bromotyrosine, which is detectable in urine. Urinary bromotyrosine levels were associated with clinical metrics of asthma control but were not correlated with other indicators of eosinophilic airway inflammation such as FeNO or blood eosinophil counts (Wedes et al., 2011). Other products of type 2 inflammation such as leukotriene E_4 are also detectable in urine (Szefler et al., 2012).

Blood eosinophil counts are elevated in subsets of asthma patients and are generally correlated, if weakly, to airway eosinophil levels. However, blood eosinophils as determined from a typical complete blood count

often exhibit substantial intrapatient variability (e.g. diurnal variation) (Szefler et al., 2012). In addition, there are many different automated blood-counting platforms in use across clinical laboratories and a lack of direct comparison across those platforms for eosinophil counts complicates the widespread implementation of blood eosinophil counts in the regulatory environment in which asthma therapies must be developed.

Soluble blood biomarkers that reflect airway inflammation with high sensitivity and specificity are desirable for the development of new asthma therapies because robust immunoassays can be developed; serum or plasma samples are easily collected, handled, and stored; and it is possible to implement immunoassays on widely available assay platforms. We have recently described the development of an assay for serum periostin, which is secreted by bronchial epithelial cells in response to IL13 and which is elevated in severe asthma patients with elevated airway eosinophils despite high-dose ICS treatment (Jia et al., 2012). Patients in a phase 2 study of lebrikizumab, a humanized mAb against IL13, with pretreatment levels of serum periostin above the median level in the study showed greater clinical benefit relative to placebo than patients with pretreatment serum periostin levels below the median (Corren et al., 2011). Clinical trials of agents targeting IL13 ± IL4 have shown significant PD effects on serum IgE levels (Corren et al., 2010, 2011) as well as levels of chemokines associated with type 2 inflammation such as CC chemokine ligand (CCL) 13 and CCL17 (Corren et al., 2011; Scheerens et al., 2011); however, the predictive value of baseline IgE, CCL13, or CCL17 was either not evident (Corren et al., 2011) or not reported (Corren et al., 2010). As peripheral blood proteins or cells may come directly from the airways as well as from other tissues in the body, it is important to interpret blood biomarker levels in the context of comorbid conditions and/or other systemic sources of those biomarkers.

A given biomarker may represent any, several, or all of the predictive, prognostic, PD, and surrogate categories. For example, sputum eosinophil percentage has been used to guide ICS dosing and this approach has been contrasted with guideline-based therapy with asthma exacerbations as an outcome measure. Patients with elevated sputum eosinophils had their ICS dose increased until sputum eosinophil levels were brought to within a certain range, whereas patients with low sputum eosinophils maintained or received lower doses of ICS. This sputum-guided approach was superior to guideline-based dosing in reducing exacerbations (Green et al., 2002; Jayaram et al., 2006). In these studies, sputum eosinophils were predictive of which patients might benefit from an increased ICS dose, prognostic for

which patients were most likely to experience exacerbations, and PD for the level of ICS dose needed to regulate airway inflammation, and decreases in sputum eosinophils in response to increased ICS doses could be viewed as a surrogate measure for decreased exacerbation rates.

3. PHARMACOKINETICS AND PHARMACODYNAMICS OF ASTHMA BIOLOGICS

3.1. Dosing

mAb therapeutics are typically administered either via the intravenous (IV) or subcutaneous (SC) route. Due to the size of antibody proteins and their instability in the gastrointestinal tract, administration via the oral route does not result in efficient absorption into the systemic circulation. There are some examples of localized delivery of protein therapeutics to the airway, which is the site of action for many of the targets under investigation for the treatment of asthma. Altrakincept is a recombinant, human, soluble IL4Rα protein that binds to IL4 and competitively inhibits its binding to IL4 receptors on cell surfaces. Two studies have shown that delivering altrakincept via nebulizer at doses ranging from 0.5 to 3 mg weekly resulted in some clinical benefit in patients with moderate persistent asthma (Borish et al., 2001, 1999). Although the site of action is presumably the airway, nebulized delivery of altrakincept did result in systemic exposure, with dose-proportional maximum serum concentrations reached at 2.5 days and a serum elimination half-life of approximately 5 days (Borish et al., 2001). Pitrakinra is an IL4 mutein that binds to IL4Rα without transducing signals, competitively blocking the access of IL4 and IL13 to the heterodimeric IL4Rα/IL13Rα1 receptor complex. It has been administered in both SC and inhaled formulations; the inhaled formulation led to low systemic exposure, although both the SC and inhaled formulations had sufficiently short half-lives to require daily (SC) or twice daily (inhaled) dosing (Burmeister Getz et al., 2009; Wenzel et al., 2007). The lower systemic exposure and shorter half-life of the IL4 mutein as compared to the soluble IL4Rα is likely due to the high expression level of IL4Rα on epithelial cells.

All monoclonal antibodies currently in development, or approved, for the treatment of patients with asthma are delivered via the IV or SC route. From a patient's convenience perspective, the SC route would be preferred over the IV route, as a visit to the doctor's office for each dose delivery would not be needed, assuming appropriate properties of the therapeutic antibody and

its formulation as well as a safety profile that allows for regulatory approval of at-home administration. Omalizumab is currently the only antibody therapeutic approved for the treatment of moderate to severe asthma and although the route of administration is SC, it needs to be administered in the physician's office, as patients must be closely observed for the occurrence of possible adverse events after administration (Cox et al., 2007; Genentech, 2010).

The route of administration (IV or SC), the dose level, and the dose frequency are all selected based on the exposure thought to result in the appropriate clinical risk/benefit ratio. When assessing these aspects during early clinical development of antibody therapeutics for the treatment of asthma, it is important to keep in mind that exposure in the lung tissue is likely lower than the exposure in the systemic circulation (which is where exposure measurements are typically collected). Preclinical studies in cynomolgus monkeys with mepolizumab and lebrikizumab suggested that there was approximately 100- to 500-fold less exposure in BAL fluid compared with the systemic circulation (Hart et al., 2001) (Scheerens et al., unpublished observations). The PK drivers for efficacy are highly dependent on the target and the mechanism of action (MOA) of the protein therapeutic. For example, to inhibit soluble cytokines such as IL4, IL5, IL9, and IL13, the conjecture is that maintaining drug exposure at a certain steady state is required for activity, assuming the rate of production of the cytokine is relatively constant over extended periods. In contrast, for therapeutics that target cellular components of the immune system directly and exert their activity via depletion of the target cells, such as benralizumab (anti-IL5Rα) or quilizumab (anti-M1prime), the activity may be more C_{max} driven, or time over a certain target, and a steady state exposure may not be required. For antibodies that block cytokine–receptor interactions by binding to cytokine receptors on the cell surface (but are nondepleting) such as AMG 317 (anti-IL4Rα), steady-state exposure is likely required, as in the case of soluble cytokine-blocking antibodies. An additional PK consideration for cytokine-receptor-blocking antibodies that bind to proteins expressed on the surface of abundant nonimmune cells (e.g. epithelial or stromal cells) is coverage of the expressed target. For a target such as IL4Rα, which is highly expressed in most tissues throughout the body, much higher doses may be necessary than for targets such as IL4 and IL13 themselves, which are expressed systemically at lower levels than their receptors, and the rate of receptor internalization and turnover further contributes to higher dose requirements. These hypotheses have to be tested throughout the clinical development of the therapeutic antibody and the initial target exposure

required for efficacy for a first-in-class therapeutic is typically estimated using information about the MOA and data from in vitro potency and preclinical efficacy studies. The final approvable/marketable dose will then be established empirically throughout a series of clinical studies and this is where PD biomarkers play a prominent role.

3.2. Pharmacodynamic Biomarkers

PD biomarkers aim to answer three main questions in clinical studies to help elucidate the clinical pharmacology of novel therapeutics: (1) does the therapeutic bind to the target, (2) does the therapeutic have biological activity consistent with the expected MOA, and (3) what is the relationship between the target binding and clinical outcomes? Understanding the answers to these questions will enable early go/no-go decisions, guide dose level and dose regimen selection, provide evidence as to the clinical pharmacological MOA of the therapeutic, and help further the understanding of disease heterogeneity. Several recent analyses of results of phase 2 clinical development studies have clearly demonstrated that success of a study depended on testing the MOA and on the confidence in the rationale being evaluated in the chosen indication (Arrowsmith, 2011; Morgan et al., 2012). Demonstrating target binding or target inhibition requires the target to be detectable in patient samples, which are generally peripheral blood samples. Because there is a clear need to investigate the pharmacology at the site of action, more specialized smaller studies are also undertaken to assess biological activity of therapeutics in the airways via collection of induced sputum or bronchial biopsies. Later in this section some examples are described to illustrate this. However, peripheral blood samples typically serve as a surrogate for evaluation of pharmacology in the airways. For benralizumab (MEDI-563), an afucosylated anti-IL5Rα antibody targeting eosinophils and basophils, studies have shown that peripheral blood eosinophils were dose dependently reduced in patients with mild asthma (Busse et al., 2010). The duration of eosinopenia was also dose dependent and can be used to optimize dosing regimens. At higher dose levels ranging from 0.3 to 3 mg/kg, eosinopenia was sustained for 12 weeks, whereas at dose levels ranging from 0.03 to 0.1 mg/kg, eosinopenia was sustained for 8 weeks. Benralizumab is an example of a therapeutic antibody targeting specific inflammatory cells where tracking of these target cells is an effective way of demonstrating target binding. Demonstrating target binding of soluble targets such as cytokines or serum IgE requires highly sensitive analytical methods. The cytokines IL4, IL5, and IL13 are present at low (picograms

per milliliter) levels in serum (St Ledger et al., 2009) and are often undetectable in a majority of patients. Binding of an anticytokine antibody to the cytokine target typically results in increased stability of the cytokine and postdose, increased levels of IL5 with mepolizumab (Stein et al., 2008), and IL13 with IMA-638, IMA-026 (Gauvreau et al., 2011), and GSK679586 (Hodsman et al., 2012), have been demonstrated. These data indicate that the target is present in the patients and that the antibodies bind to their respective targets. The increase in total cytokine levels is due to decreased clearance of the cytokine–antibody complex. Target binding PD assays can also shed light on the production rate of the cytokine and clearance mechanism and understanding these aspects of the target in human disease will greatly aid in trial design, particularly dose level and dose regimen selection. An interesting example of elucidating the clearance mechanism of IL13 in asthmatics comes from the studies conducted with IMA-638 and IMA-026. These anti-IL13 antibodies have distinct binding epitopes: IMA-638 blocks IL13 binding to IL4Rα, while IMA-026 blocks IL13 binding to IL13Rα1 and IL13Rα2. Total IL13 levels were approximately 10-fold higher following dosing with IMA-026 than with IMA-638, and the authors concluded that IMA-026 blocks the very efficient physiological clearance mechanism of IL13 via the IL13Rα2 pathway leading to higher total IL13 levels (Gauvreau et al., 2011; Kasaian et al., 2011). This observation may partially explain the decreased efficacy observed with IMA-026 relative to IMA-638 and supports a physiological role of IL13Rα2 as a nonsignaling decoy receptor for IL13. Another, nonmutually exclusive explanation of the relative lack of efficacy for IMA-026 may be that IMA-026 has lower affinity for IL13 than does IMA-638 (Kasaian et al., 2008).

For omalizumab, which targets soluble IgE, both a total IgE and free IgE assay were developed to fully characterize PK/PD correlation in patients with asthma and the omalizumab dose level and dose regimen were selected based on target PD levels. Omalizumab is administered SC and dose level and frequency have been selected based on obtaining free IgE levels below the clinical target of 25 ng/ml, which initial studies had indicated was associated with clinical benefit (Hochhaus et al., 2003). Subsequently, an individualized dosing table was created based on baseline IgE and patient's body weight, indicating that patients are to receive omalizumab either every 2 weeks or once a month at dose levels ranging from 150 to 375 mg (Boulet et al., 1997; Casale et al., 1997; Holgate, Buhl, et al., 2009; Holgate, Smith, et al., 2009). More recent mechanistic PK/PD modeling analyses have indicated that the dosing table is close to optimal for most patients to achieve

the desired target free IgE levels but that those levels could possibly also be achieved in some patients currently outside the margins of the approved dosing table (Lowe et al., 2009).

The PD biomarker examples described above illustrate how target binding assays can increase confidence in a novel therapeutic in early clinical studies and how they can support dose selection. It is equally important to demonstrate that target binding results in pharmacological activity consistent with the expected MOA. MOA-related biomarkers are selected based on the understanding of the biology mostly gathered from in vitro and preclinical studies. Although preclinical animal models of asthma are not necessarily fully predictive of human biology especially regarding clinical end points, disease heterogeneity, or the chronic aspects of the disease, they are useful tools for studying particular pathways involved in the disease and are used to identify MOA-related PD biomarkers to be evaluated in the clinic. For example, IL13 can induce the production of the chemokines CCL13 and CCL17 in airway cells and modulation of systemic levels of CCL13 and CCL17 has been shown with lebrikizumab (Corren et al., 2011; Scheerens et al., 2011). Similarly, FeNO is immediately downstream of IL13 as the enzyme NOS2 (also known as iNOS) is transcriptionally regulated by IL13 in bronchial epithelial cells (Jiang et al., 2009). Lebrikizumab reduced FeNO as early as 4 weeks after the first dose and levels remained suppressed for the duration of the treatment (Corren et al., 2011). Reduction of serum chemokines and FeNO following lebrikizumab administration established evidence of pharmacology both systemically and at the site of action and indicated onset of action following one dose administration. Modulation of FeNO has also been demonstrated with other therapeutics targeting the IL4 and IL13 pathways. FeNO levels were clearly reduced in patients with mild to moderate asthma treated with altrakincept (Borish et al., 1999) and pitrakinra (Wenzel et al., 2007), with both molecules delivered topically to the airway as well as by GSK679586 following IV administration (Hodsman et al., 2012). In contrast, no significant effects on FeNO levels were observed following treatment with AMG 317 also targeting the IL4 and IL13 pathways (Corren et al., 2010). Pharmacological activity of AMG 317 was established in that study via a dose dependent decline in serum IgE, suggesting adequate dosing. Low predose levels of FeNO (likely in part due to background ICS treatment) in the two highest AMG 317 dose groups likely confounded the ability to measure effects on FeNO. Another example highlighting the importance of predose biomarker levels is illustrated in the study with lebrikizumab. Larger effects of IL13 blockade were observed

on both FeNO and serum periostin levels in patients with elevated baseline FeNO and serum periostin levels when compared to patients with lower baseline levels of these biomarkers (Corren et al., 2011; Scheerens, Arron, et al., 2012). Therefore, in a disease with heterogeneous activity levels of the targeted pathway, a PD biomarker needs to be present at baseline (predose) in sufficient quantities to allow for assessment of modulation by the therapeutic. Although FeNO and serum periostin levels were elevated in the examples above, all that is required is the presence of a sufficiently dynamic window. In healthy volunteers even with mean serum IgE levels below 100 IU/ml, a dose-dependent reduction of serum IgE was observed with quilizumab, an anti-M1prime antibody targeting IgE-specific B cells (Scheerens, Putnam, et al., 2012). Thus, patient selection and ensuring that appropriate baseline characteristics allow for assessment of pharmacological activity are critical to interpret how PD biomarkers inform about the pharmacological activity of novel therapeutics.

In addition to direct target binding assays and MOA PD biomarker assays, there are several biomarkers associated with asthma in general that tend to get measured in many clinical studies irrespective of the linkage to the therapeutic target. Examples of these biomarkers are serum IgE, eosinophils, and FeNO. FeNO is widely accepted as a surrogate biomarker of eosinophilic airway inflammation in general and in fact has been used effectively in dose titration studies with ICSs (Powell et al., 2011). Interestingly, studies with mepolizumab have demonstrated that despite significant reduction in airway eosinophilia, no reduction in FeNO was observed, suggesting that FeNO levels may be dissociated from eosinophils and require other components of the inflammatory cascade to be inhibited as well (Haldar et al., 2009).

The PD of peripheral blood eosinophils in anti-IL5 and anti-IL13 studies present an interesting illustration of the varying effects of IL5 and IL13 on eosinophil biology. IL5's dominant function with respect to eosinophils is to promote eosinophil hematopoiesis and mobilization, while IL13's dominant function with respect to eosinophils is to act on target tissues to induce the expression of eosinophil chemoattractants such as eotaxins. IL5 blockade with mepolizumab (Flood-Page et al., 2007; Haldar et al., 2009; Nair et al., 2009) or reslizumab (Busse et al., 2011) dramatically reduces the levels of circulating and sputum eosinophils, presumably by cutting off their production. However, anti-IL5 only partially depletes eosinophils in bronchial tissue (Flood-Page et al., 2003), suggesting that other factors such as granulocyte-macrophage colony-stimulating

factor and eotaxins may be sufficient to sustain tissue-resident eosinophils. In the case of IL13 blockade with lebrikizumab, levels of the eosinophil chemoattractant CCL13 were significantly decreased, while levels of circulating eosinophils exhibited a modest increase (Corren et al., 2011). Although the effect of IL13 blockade on tissue eosinophils has not yet been assessed, these findings, taken together, support the model that IL5 promotes eosinophil hematopoiesis, while IL13 promotes eosinophil trafficking to inflamed tissues.

3.3. PD Biomarkers and Linkage to Clinical End Points

PD biomarkers play a clear role in ensuring that the pharmacology of novel therapeutics is adequately tested in early clinical studies. For most therapeutics currently in development, direct target, MOA, and general asthma inflammatory biomarkers are now routinely being measured. The remaining question concerns the linkage between the PD biomarkers and clinical end points. The underlying molecular pathways driving end points such as asthma exacerbations, FEV1, and symptoms are extremely complex, likely multifactorial, and different for each end point. PD biomarkers such as FeNO, cytokines, eosinophils, and IgE are each likely to play some part in the pathophysiology underlying the clinical end points of asthma, but they do not tell the whole story. Continued evaluation of the mechanisms of novel therapeutics in different patient subsets will help further the understanding of the molecular pathways underlying the symptoms characteristic of asthma. Because a direct link between the PD biomarkers and clinical end points generally applied to asthma clinical trials has not been established to date, caution should be exercised when using PD biomarkers to predict clinical outcome and to compare different therapeutics directly. Using PD biomarkers to compare different therapeutics and predicting clinical outcome can only be effectively done when comparing therapeutics blocking the same target, as may be the case with follow-on biologics.

4. CLINICAL STUDY DESIGN FOR ASTHMA BIOLOGICS

4.1. Clinical Study Phases

An exploratory investigational new drug (IND) approach has recently been pioneered in oncology (Doroshow & Parchment, 2008). These phase 0 or microdosing studies are intended to characterize the PK of chemical lead candidates or the imaging of specific human drug targets. Microdosing

studies are not intended to produce a pharmacologic effect. Doses are limited to less than one-hundredth of the dose predicted to produce a pharmacologic effect in humans (based on preclinical data) or to a dose of less than 100 μg, whichever is lower. Exploratory IND-enabling preclinical safety requirements for microdosing studies are substantially less than those for the conventional IND approach (Hughes et al., 2004). It is likely that in the foreseeable future this approach will be applied in nononcology indications.

Phase 1 trial design for a novel therapeutic with potential to impact asthma follows standard principles for the testing of a novel therapeutic. Safety is the primary consideration for phase 1 and is the benchmark by which stages of experience can be evaluated. TGN1412 is a recent example where a biologic investigational therapeutic failed in phase I due to toxicity that could potentially have been predicted from the MOA of the agent. TeGenero's first product was TGN1412, a CD28 "super-agonist" intended to stimulate regulatory T cells, which caused near-fatal inflammatory side effects in its first trial in healthy human subjects in March 2006 (Dowsing & Kendall, 2007). The TeGenero experience was followed by a revisiting of the factors that determine successful phase 1 conduct and first dose determination particularly for biologics (Brennan et al., 2010; Danilenko & Wang, 2012; Lowe et al., 2010). It also created a new definition of high-risk product for regulatory assessment. Medicinal products are defined as potential high-risk medicinal products when there are concerns that serious adverse reactions in first-in-man clinical trials may occur. These concerns may be derived from particular knowledge or uncertainties on (1) the mode of action, (2) the nature of the target, and/or (3) the relevance of animal models (EMA, 2007).

An evaluation of the potential impact of the intervention determines if a population with the greatest reserve to recover fully from any adverse event such as healthy volunteers or patients with mild allergic rhinitis (who are allergic but without asthma) is required or a population with established disease where the risk/benefit ratio might be more appropriate to obtain safety experience. For example, GSK679586, MEDI-528, tralokinumab, and omalizumab were first tested in phase 1 studies in healthy volunteers or allergic rhinitis patients (Catley et al., 2011). Therapeutics initially developed for other indications such as keliximab (anti-CD4) (Kon et al., 1998; Yocum et al., 1998), daclizumab (anti-CD25) (Busse et al., 2008; Vincenti et al., 1998), and golimumab (anti-TNFα) (Wenzel et al., 2009; Zhou et al., 2007) had already undergone safety testing in appropriate populations for other indications and the first exposures for asthma were in moderate to

severe asthma patients. In addition, healthy volunteers can be more rapidly recruited compared to patients with particular disease states.

Healthy volunteers have been used to quantify potential bronchodilator effects or protection from bronchoconstrictor effects (Lecaillon et al., 1999; Ostrom, 2003; Youlten et al., 1988). When the targeted pathway is present in both healthy and diseased individuals, short-term exposures of large molecules may allow the assessment of PD effects in healthy volunteers (Mascelli et al., 2007; Zhou et al., 2012). Both omalizumab and the recent biosimilar CMAB007 were introduced by using IgE as the PD end point in healthy subjects or subjects with seasonal allergic rhinitis for safety and (Casale et al., 1997; Zhou et al., 2012). While mainly directed at small molecules, a therapeutic index can be obtained for side effects that are demonstrable in healthy volunteers including effects on cortisol, potassium, and glucose (Guhan et al., 2000; Uhl et al., 2002; van den Berg et al., 1998).

Phase 1 studies must be initiated with single-dose exposures. Considerations must be given for a suitable interval between or within individuals or groups of subjects before dose escalation can commence depending on the study design, the properties of the drug and the risk assessment of the molecule (Mascelli et al., 2007).

Phase 2A study designs can be thought of as providing proof of activity or proof of concept. While these two concepts have been used interchangeably, proof of activity may be considered a first exploration and can encompass a smaller trial in patients with the disease or a surrogate for the disease in healthy volunteers. Other labels such as "pilot study" have been used to distinguish an initial attempt to demonstrate efficacy and safety in order to provide a justification for greater investment for a therapeutic (Moore et al., 2011).

The allergen challenge and the exercise bronchoconstriction models have been the most frequently used study designs to evaluate potential for impacts on efficacy in asthma. The allergen challenge model is thought to have a high negative predictive value for efficacy in the clinic (O'Byrne et al., 2009). However, it may be more of a model of an acute exacerbation as the late-phase response is initiated by an acute exposure to allergen. The difficulties with the model are that careful standardization of technique is required to ensure the ability to quantify the early- and late-phase response in a reproducible way, and only approximately half of screened subjects demonstrate a late-phase response. Using subjects as their own control in cross-over designs has been very successful for small molecules

with short half-lives but the long half-life of monoclonal antibodies often precludes this approach. A parallel group design requires more subjects than is typically used for a cross-over study to detect a significant inhibition of the late-phase response. The negative result of the impact of mepolizumab on allergen challenge in a phase 1 safety study sparked a considerable debate as to whether the study was sufficiently powered to detect a small effect or should it have been considered a safety study only (O'Byrne et al., 2001). Subsequent trials of mepolizumab have only demonstrated benefit in subjects with eosinophilic asthma as defined by 3% or more eosinophils in induced sputum (Haldar et al., 2009; Nair et al., 2009) and it may be that the typical numbers of all comers enrolled in an allergen challenge model did not include sufficient numbers of patients with eosinophilic disease.

The exercise-induced bronchoconstriction (EIB) model may be a useful proof-of-activity study. EIB is a common diagnosis that limits physical activity in many individuals. Its prevalence is estimated as 50–90% in patients with asthma, 11–50% in athletes overall (whether or not they also have overt asthma), and 10–15% in the general population (Philip et al., 2007). Amelioration of EIB can be used as a stand-alone claim for clinical benefit (FDA, 2002) and has also been used to help define dose-ranging responses for montelukast (Bronsky et al., 1997). A recent pilot study demonstrated that measurements of sputum eosinophil percentage may be useful in predicting the magnitude and temporal response of EIB to different dose levels of ICS (Duong et al., 2008). While it is considered a useful model to test antiasthma activity, the positive predictive value of this approach for other asthma end points is not fully understood. Patients recruited into studies of EIB have demonstrated EIB that improves after randomization (as indicated by their responses to exercise after receiving placebo) (Philip et al., 2010). Large placebo response improvement is not easily explained; however, some of the observed improvement may have occurred simply through "regression to the mean," despite the study requirement for two documented episodes of EIB before randomization. The repetition of exercise may have also played a role, in that it has long been known that airways become temporarily refractory to bronchoconstriction after exercise (Edmunds et al., 1978). However, while a repetition of exercise might be capable of eliciting a long-lasting form of refractoriness, the refractoriness to EIB has generally been shorter than the interval used to separate testing.

MEDI-528 (anti-IL9) was tested using an EIB model as proof of activity alongside other tests of safety (Parker et al., 2011). The original design included three cohorts (50 mg, 100 mg, and 200 mg MEDI-528), each with 18 subjects randomized 2:1 to receive MEDI-528 or placebo. Sample size calculations were based on two-sample *t*-test of the reduction in the maximum decline of FEV1 after exercise challenge testing. The power to detect a statistically significant difference in the maximum decline in FEV1 was greater than 80% based on an assumption of a 20% fall in the placebo group and 65% reduction in the maximum decline of FEV1 in the combined group (36 subjects) on active treatment versus placebo (18 subjects). This study was halted prematurely due to a serious adverse event (SAE) in an asymptomatic MEDI-528-treated subject who had an abnormal brain magnetic resonance imaging (required for evaluation based on preclinical findings) that was found to be an artifact on further evaluation. However, only 11 subjects were enrolled before the study was stopped prematurely, which allowed only two evaluable subjects on placebo and seven subjects in the active group. While the data look promising as a "pilot study" or "proof of activity" study, the molecule was subsequently tested in a larger proof-of-concept clinical study that was negative for the primary end point (Oh et al., 2012).

A proof-of-concept study is typically conducted in the clinic with the target population with a view that the subsequent phase 3 studies would be confirmatory. At a simplistic level, a phase 2 study can be conducted with the smallest number of subjects that would give confidence such that when repeated in replicate studies the primary end point would be confirmed with some degree of certainty. This can be justified with a sample size through estimations using the assumption of 80% power, known variability of the end point, estimated treatment effect size expected, type 1 error rate assumptions and a study design that controls for confounding factors (such as blinded run-in and strict entry criteria for a stable baseline). The confidence limits are estimated such that the ability to detect a treatment effect can be defined. The primary end point is declared to manage multiple testing where all the data from the secondary end points are evaluated to look for impacts across a range of asthma end points. The sample size and the operational limits of the study are assessed and it is likely that feasibility trumps a desire for a larger more "robust" study. It should be noted that phase 3 studies do not always have to be conducted to 90% power as 80% may be sufficient to confirm clinically important effects (Kay, 2007, p. 136). The objectives for phase 2

proof-of-concept studies are primarily to establish if the treatment effect is clinically meaningful and the risk–benefit profile is suitable to invest in larger confirmatory studies. These studies can include dose ranging or "finding" and the Food and Drug Administration recommends including dose exploration throughout all phases of development (Temple, 2008). Given the intrapatient variability of many asthma outcome measures, establishing a dose response can be challenging, and often, additional studies are required to establish a minimal effective dose or an optimal dose (Young, 2012, p. 182).

Phase 3 trials are designed to confirm the final to-be-marketed form of the product, the final dose and/or regimen, and the population(s) and to establish sufficient risk–benefit experience for an approval in a specific indication. In asthma, adolescents and adults are studied together as appropriate based on a safety assessment. Pediatric development has to be considered as soon as initial adult PK and safety data are available and a clinical development plan developed for the entire pediatric age range. While it may be appropriate to initiate treatment in the very young, considerations for the diagnosis of asthma and monitoring of treatment response have to be made for the 0–6 year age group. The challenge of asthma in the elderly is now being considered and there is relatively little experience for biologics in asthma for the over 65 year age group (Reed, 2010). Considerations for drug–drug interactions have to be made but usually these can be gained from the safety assessment of the phase 3 population. The size of the phase 3 population is largely driven by considerations of safety to allow a large enough denominator to confidently exclude less-than-common adverse events. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline for 1500 patients remains the standard benchmark by which a minimum population size should be considered for an asthma treatment in a condition that is largely considered non–life threatening (ICH, 1994). Electrocardiographic monitoring is an integral component of the clinical assessment of cardiac safety of all compounds in clinical development. There has been limited published information on QT interval changes secondary to therapeutic proteins. In theory, biologic therapies may affect cardiac electrical activity either directly or indirectly. The Cardiac Safety Research Consortium has published a guidance including possible approaches to consider for the clinical evaluation of drug-induced QT prolongation in development programs of therapeutic proteins (Rodriguez et al., 2010).

4.2. Outcome Measures

Clinical trial outcome measures for asthma have traditionally been based on bronchodilation and FEV1 is the established primary end point for asthma efficacy (Shen et al., 2011). FEV1 is repeatable to within 120–150 ml with a single spirometry session and thus it is an objective measure within certain operational limits, which makes it an attractive end point (Enright et al., 2004). The disadvantages are that certain quality controls need to be in place to ensure that the data collected is reliable (Malmstrom et al., 2002). The minimally important change in FEV1 is not fully established. Increments of less than 8% relative change (or <150 ml absolute change) are likely to be within measurement variability and may justify 120 ml as a suitable minimally important difference. However, patients cannot estimate their FEV1 and it correlates poorly with symptoms or exacerbations. One study determined an average minimal perceived improvement in FEV1 as 230 ml based on analysis of mild to moderate asthmatics in a placebo-controlled trial (Santanello et al., 1999). Severe asthmatics may have irreversible disease and consequently may be excluded from trials that typically enroll using acute bronchodilation as an entry criterion (Robinson et al., 2012). Asthma symptoms correlate poorly with the level of airway obstruction as determined by the FEV1 and peak expiratory flow (Teeter & Bleecker, 1998). Following treatment, subjective improvement in asthma symptoms may occur without improvement in the level of airway obstruction. These results support the recommendation to measure airway obstruction objectively when assessing adult patients with chronic asthma. *However, the relationship among asthma end points is complex, preventing simple definitions of asthma control and individual responses.* Variability may be reduced when measurements at multiple time points are taken and averaged. Thus, changes in a single measurement at any one time point do not adequately describe the results of asthma therapy in an individual patient, and consequently, it may be difficult to accurately predict the outcomes of therapy (Shingo et al., 2001; Zhang et al., 2002).

Furthermore, patients who show a robust improvement in pulmonary function do not necessarily show a similar change in daily symptom scores or β-agonist use. Measures of changes in pulmonary function, which may be thought of as objective, correlated poorly with patient-reported (subjective) measures of asthma. Lung function is not associated with any measure of asthma control in patients with severe fixed airflow obstruction. FEV1 correlates well with asthma symptoms in difficult asthma patients with poor

control but not when control improves. This loss of relationship is due to subjects with severe fixed airflow obstruction, where good subjective control does not exclude the presence of significant obstruction (Aburuz et al., 2005).

Another approach that may be usefully employed in phase 2 to help ensure that a trial is informative and militate against a large placebo response is to include a blinded run-in. If subjects are initiated on blinded placebo treatment and can be assessed for stability of asthma prior to randomization, it may help mitigate the impact of a large placebo response typical of many asthma trials but this has not be widely employed (Barnes et al., 2012).

The aim of asthma treatment is to minimize symptoms, optimize lung function, and prevent exacerbations. Increasingly, there has been recognition of the importance of measuring the patient perspective and of the poor correlation between lung function and inflammation and symptoms. Tools to measure symptoms seem relatively straightforward but no single instrument has been endorsed as either essential for asthma trials or able to be applied across the spectrum of asthma severity (Krishnan et al., 2012).

There has been a shift to the assessment of "asthma control." This is a summary term that implies a global assessment of symptoms, reliever use, lung function, and the frequency/severity of exacerbations (Reddel et al., 2009). There has been an attempt to standardize the definition of both asthma control and an "exacerbation" (Fuhlbrigge et al., 2012; Reddel et al., 2009). The Asthma Control Composite (ACC) tool was developed using real-time diary-based data from trials of omalizumab to predict treatment response in terms of asthma control. The ACC tool accurately reflects asthma control in moderate to severe asthma patients eligible for biological therapy. Unlike the Asthma Control Test (ACT), which has not been validated in this patient population, the ACC shows promise as an asthma control assessment tool in patients with moderate to severe asthma (Harris et al., 2011).

A recent asthma end points assessment conducted by an NIH workshop group has identified standardized outcomes where possible (Busse et al., 2012). *Asthma clinical research will highly benefit from standardization of major outcomes in terms of definition and assessment methodology, as standardization will permit useful comparisons across interventional or observational studies and will allow more effective data sharing.* "Asthma severity" is yet another concept that requires careful consensus to help achieve uniformity across study populations. The profound unmet need in severe asthma requires new approaches (Blakey & Wardlaw, 2012; Gibeon & Chung, 2012; Hashimoto & Bel, 2012; Robinson et al., 2012; Wenzel, 2012). A personalized medicine approach

can more effectively direct therapeutics to those who stand to get the greatest benefit from them and avoid prescribing them to those who do not derive clinically meaningful benefit or who could be harmed. Biological agents administered by parenteral route are likely to be given as add-on therapy for patients who are found to have severe asthma despite application of an inhaled steroid and at least a second controller (Heaney & Horne, 2012). This has given rise to the concept of a "biologic eligible" population.

4.3. Biomarker-Guided Clinical Trial Design

A large and growing number of investigational biologic therapeutics has entered clinical development for the treatment of asthma and have been extensively reviewed elsewhere (Catley et al., 2011; Holgate, 2012); rather than recapitulate the study design and outcomes of each, we will briefly consider selected examples here (a full listing of the biologic agents, their targets, and mechanisms of action is discussed in Table 1.1). The objective of this section is to illustrate the concepts of using predictive biomarkers to guide patient selection and selection of outcome measures best suited to interrogate whether a given agent has clinical activity in the target patient population.

4.3.1. Therapies Targeting IgE

Omalizumab is the only approved biological treatment for asthma. As omalizumab prevents the binding of IgE to FcεRI on mast cells and basophils, its activity is likely to be most relevant in asthma patients whose disease is driven by IgE, i.e. allergic asthmatics. As such, it is indicated for asthma patients with a history of allergy, skin prick test positivity to common aeroallergens, and/or elevated serum levels of allergen-specific IgE. Thus in one sense, omalizumab is a personalized therapy, as a substantial proportion of moderate to severe asthma patients are ineligible for omalizumab treatment because they lack evidence for allergic disease. Because of PK considerations, as described above, omalizumab eligibility is further restricted by its dosing table, which requires that the total serum IgE be within a particular weight-adjusted range so that effective target neutralization is possible at selected doses (Genentech, 2010). It was approved on the basis for its ability to reduce exacerbations and the overall safety profile seen at the time of filing (EMA, 2009; FDA, 2009). Omalizumab was initially dosed in healthy volunteers and then proof of activity was achieved by showing a treatment effect using the allergen challenge model. Consistent with its MOA, omalizumab blocks both the early-phase allergic response (EAR) and late-phase

Table 1.1 Investigational biologic asthma therapies discussed in this chapter

Agent	Target	Mechanism of action
Omalizumab	IgE	mAb against IgE that blocks binding of soluble IgE to FcεRI
CMAB007	IgE	mAb against IgE that blocks binding of soluble IgE to FcεRI
Quilizumab	IgE (M1 prime epitope)	mAb against M1 prime epitope of IgE that depletes B cells expressing transmembrane IgE
Altrakincept	IL4	Soluble IL4Rα-Fc fusion protein that blocks binding of IL4 to endogenous IL4Rα
Pitrakinra	IL4, IL13	IL4 mutein that blocks binding of IL4 and IL13 to IL4Rα
AMG 317	IL4, IL13	mAb against IL4Rα that blocks IL4 and IL13 binding to IL4Rα
Lebrikizumab	IL13	mAb against IL13 that blocks signaling by IL13 through the IL4Rα/IL13Rα1 receptor complex
IMA-638	IL13	mAb against IL13 that blocks signaling by IL13 through the IL4Rα/IL13Rα1 receptor complex
Tralokinumab	IL13	mAb against IL13 that blocks signaling by IL13 through the IL4Rα/IL13Rα1 receptor complex
GSK679586	IL13	mAb against IL13 that blocks binding of IL13 to IL13Rα1 and IL13Rα2
IMA-026	IL13	mAb against IL13 that blocks binding of IL13 to IL13Rα1 and IL13Rα2
Mepolizumab	IL5	mAb against IL5 that blocks binding of IL5 to IL5Rα
Reslizumab	IL5	mAb against IL5 that blocks binding of IL5 to IL5Rα
Benralizumab	IL5Rα	mAb against IL5Rα that depletes cells expressing IL5Rα
MEDI-528	IL9	mAb against IL9 that blocks binding of IL9 to IL9R
Etanercept	TNFα	Soluble TNFRII-Fc fusion protein that blocks binding of TNFα to endogenous TNF receptors
Golimumab	TNFα	mAb against TNFα that blocks binding of TNFα to TNF receptors
Keliximab	CD4	mAb against CD4 that depletes CD4+ T cells
Daclizumab	CD25	mAb against CD25 that depletes activated (CD25+) T cells

allergic response (LAR) in bronchial allergen challenge studies. Two separate trials were positive for inhibition of early- and late-phase reaction when omalizumab was tested at a high IV dose versus placebo (Boulet et al., 1997; Fahy et al., 1997). It was then tested in adult and adolescent patients with moderate to severe persistent perennial allergic asthma using daily ICSs (or daily OCSs) to look for a change in day time and nocturnal asthma symptom scores using placebo, high-, and low- dose IV omalizumab (Milgrom et al., 1999). The study met its primary end point and the trial design was the basis of subsequent phase 3 pivotal studies using an SC formulation and a dosing table (Busse et al., 2001; Soler et al., 2001). Asthma exacerbations were counted as events during a corticosteroid reduction period and perhaps the most striking observation of efficacy was noted in the protection afforded during OCS reduction relative to the smaller impact on FEV1 after the steroid-stable phase. Ultimately, omalizumab has been tested in large placebo-controlled studies to quantify the benefit in terms of exacerbation rate reduction in patients despite taking high-dose ICSs and LABAs (Hanania et al., 2011). The benefit of omalizumab has also been tested as a corticosteroid-sparing agent in smaller proof-of-concept trials (Holgate et al., 2004).

However, not all patients, even restricted to allergic asthmatics, demonstrate clinical benefit from omalizumab. Within omalizumab studies, further subsets of patients with enhanced clinical benefit have not yet been identified. Considering that IgE can act both upstream (via FcεRI-mediated mast cell degranulation) and downstream (via the induction of isotype switching to IgE in activated B cells) of IL13, it will be of interest to assess the degree to which predictive biomarkers that identify patients with enhanced clinical benefit from IL13-blocking therapies (discussed below) may identify subsets of patients with enhanced clinical benefit from omalizumab, including the degree of overlap, if any, between "responder" populations.

Quilizumab (anti-M1prime), an mAb against the M1prime epitope of transmembrane IgE that is transiently expressed on B cells between the time of isotype switching and differentiation into IgE-secreting plasma cells, is currently in phase 2 studies in asthma patients. Like omalizumab, the target of quilizumab is IgE; however, rather than directly targeting the binding of soluble secreted IgE to FcεRI, quilizumab is believed to inhibit the production of new IgE through the selective depletion of newly formed IgE-producing B cells (Brightbill et al., 2010). In phase 1 studies, quilizumab treatment led to mean reductions of approximately 25% of total serum IgE (Scheerens, Putnam, et al., 2012). In a phase 2a allergen challenge study, quilizumab

reduced both EAR and LAR relative to placebo and, consistent with its proposed MOA, prevented allergen-challenge-induced increases in allergen-specific IgE (Gauvreau et al., 2012). It remains to be determined whether this will translate to clinical benefit in more severe asthma patients but, if effective, quilizumab has the potential to produce sustained reductions in systemic IgE levels without the dosing constraints associated with omalizumab.

4.3.2. Therapies Targeting IL5 and its Receptor

A phase 2 study of mepolizumab (anti-IL5) in moderate asthmatics incompletely controlled on ICS but not otherwise selected according to phenotype showed substantial reductions in blood and sputum eosinophils but only a partial reduction in bronchial mucosal eosinophils. The study failed to show any benefit in terms of lung function or asthma control as assessed by the asthma control questionnaire (ACQ)(Juniper et al., 1999) but did show a nonsignificant trend toward reduction in exacerbation rates (Flood-Page et al., 2007). Two subsequent studies that enrolled only patients with greater than 3% sputum eosinophils and multiple exacerbations in the previous year found that mepolizumab could produce a significant reduction in exacerbation frequency (Haldar et al., 2009; Nair et al., 2009). A phase 2 study of reslizumab (anti-IL5) in moderate to severe patients with 3% or more sputum eosinophils showed significant improvements in FEV1 and trends toward improvement in ACQ score and exacerbation rates, particularly in patients with concomitant nasal polyposis, a frequent comorbidity of eosinophilic airway disease (Castro et al., 2011). Benralizumab (anti-IL5Rα), which is cytotoxic to eosinophils, was investigated in a single-dose study of patients with severe eosinophilic asthma who presented with exacerbations. The overall rate of exacerbations over the 24 weeks following treatment was significantly reduced in that study and significant effects on blood eosinophil counts were observed as long as 12 weeks following dosing (Molfino et al., 2012). Taken together, these studies show that targeting IL5 can substantially reduce eosinophils in asthma patients and reduce asthma exacerbations. However, to demonstrate this effect, patients who have both increased airway eosinophilia and a history of asthma exacerbations should be selected, as prior exacerbations are generally prognostic for future exacerbations (Miller et al., 2007).

4.3.3. Therapies Targeting IL4, IL13, and Receptors

Outcomes from several phase 2 studies of biologic agents targeting IL4, IL13, or their receptors have been reported. Several allergen challenge studies

with effects on LAR have been reported for agents including pitrakinra (Wenzel et al., 2007), anrukinzumab (IMA-638), IMA-026 (Gauvreau et al., 2011), and lebrikizumab (Scheerens et al., 2011). While differing in their precise mechanisms of action and routes of administration, each agent is intended to block IL13 signaling through the IL4Rα/IL13Rα1 complex, while pitrakinra should also block IL4 signaling by competitively binding to IL4Rα. In general, IL13-blocking agents produced comparable placebo-adjusted effects on LAR, although IMA-026 failed to demonstrate benefit. This lack of benefit was attributed to IMA-026's ability to block IL13 binding to the IL13Rα2 decoy receptor, which may serve as a physiological sink for excess IL13 (Kasaian et al., 2011), but could also be in part due to IMA-026's lower affinity for IL13 (Kasaian et al., 2008). Patients with baseline levels of blood eosinophils, IgE, or serum periostin above the median exhibited greater placebo-adjusted LAR reduction from lebrikizumab than patients with levels of these biomarkers below the median (Scheerens et al., 2011). Patients with common single nucleotide polymorphisms (SNPs) in the *IL4RA* gene associated with incident asthma tended to have a greater LAR improvement than those with wild-type variants at that locus (Slager et al., 2010). While consistent with the biology of these biomarkers and suggestive of a subset of mild asthma patients in whom IL13 is a more dominant player in allergic responses, these studies were small and underpowered to detect significant effects in those subsets, hence they should be interpreted with caution.

While demonstrating efficacy in a bronchial allergen challenge study provides mechanistic support that IL13 (±IL4) blockade can ameliorate the loss of lung function during a provoked episode of airway inflammation, allergen challenge studies are typically conducted in mild asthmatics not on ICSs. To show benefit under more "real-world" conditions in the target patient population for biologic therapies, larger phase 2 randomized placebo-controlled studies of at least 12 weeks of pitrakinra (Otulana et al., 2011; Slager et al., 2012), AMG 317 (Corren et al., 2010), lebrikizumab (Corren et al., 2011), and tralokinumab (Piper et al., 2012) were conducted in several hundreds of moderate to severe asthmatics per study who remained symptomatic despite ICS therapy. The pitrakinra, AMG 317, and tralokinumab studies were dose ranging, while the lebrikizumab study examined a single dose. The priorities of the specified outcome measures differed across the studies, but end points common to all three studies were FEV1, exacerbations, and asthma control as defined by ACQ. While both lebrikizumab and tralokinumab demonstrated modest improvements vs

placebo for FEV1 in all comers, AMG 317 did not. However, in a posthoc analysis of the AMG 317 study, the most symptomatic patients (as defined by those with the highest tertile of ACQ scores at baseline) in the highest dose arm showed a trend toward significant FEV1 improvement. In the lebrikizumab study, a prespecified analysis subdividing patients according to baseline serum periostin levels greater than vs less than the median showed that "periostin-high" patients had a much greater FEV1 benefit than "periostin-low" patients. Similar enrichment for FEV1 benefit from lebrikizumab was observed when patients were subdivided according to baseline FeNO or blood eosinophil levels. In the tralokinumab study, a posthoc analysis of a subset of patients with available data for sputum IL13 levels found that patients with 10 pg/ml or more of IL13 in sputum at baseline had greater FEV1 benefit than patients with less than 10 pg/ml of IL13 in sputum. None of the studies showed significant benefit for ACQ, but a feature common to each study was enrollment criteria specifying relatively poor asthma control (ACQ ≥ 1.5 at study entry) and substantial ACQ improvements were generally observed in the placebo arms of the studies. In the pitrakinra study, enhanced benefit in terms of reduced exacerbation rate was observed in patients with elevated blood eosinophils, elevated FeNO, or SNPs in *IL4RA*, although these subsets, as defined, exhibited little overlap. None of the studies showed significant benefits for severe asthma exacerbations in the primary analyses, although they were underpowered for that outcome measure because of the short duration and relatively small size of each study. Although the treatment period for lebrikizumab was 20 weeks, PD effects on FeNO and IgE were observed for as long as 32 weeks, and a statistically significant reduction in severe exacerbations was observed in the lebrikizumab vs placebo arm when considering the full 32-week observation period (McClintock et al., 2012).

Taken together, the clinical studies targeting IL13 illustrate that patient selection and outcome selection are important determinants of the ability to demonstrate clinical benefit. The multiple bronchial allergen challenge studies in mild allergic asthmatics show that IL13 is a key mediator of the late-phase allergen response. The studies examining the benefit of IL13 blockade in patients who are not well controlled despite ICS therapy show that lung function as measured by FEV1 can be impacted, but this effect is predominant in patients with evidence of "type 2 high"/eosinophilic asthma as determined by high serum periostin, FeNO, blood eosinophils, or sputum IL13 levels, whereas patients with lower levels of periostin, FeNO, or sputum IL13 levels do not exhibit substantial benefit from anti-IL13.

It has been difficult to show benefit on composite outcome measures such as ACQ in this population, which may be in part attributed to a large placebo response, lack of sensitivity of the instrument in this population, regression to the mean, and/or improved adherence to background ICS therapy in both the active and placebo arms of the studies. As the major health economic impact of poorly controlled moderate to severe asthma comes from health care utilization associated with severe exacerbations (Braman, 2006; Jackson et al., 2011), future studies of anti-IL13 therapies should be of appropriate size and duration to accurately measure whether there is a significant impact on exacerbations.

4.3.4. Biologic Therapies in Asthma Targeting IL9 and TNFα

Compared to the biologies of IL4, IL5, and IL13, the biology of IL9 and the role it plays in the pathogenesis of human asthma is less clear. Increased IL9 expression was detected in the airways of patients (Erpenbeck et al., 2003; Shimbara et al., 2000; Ying et al., 2002) and some data suggest that IL9 may play a role in the trafficking of mast cells to the airways (Jones et al., 2009). As discussed above, MEDI-528 (anti-IL9) was investigated in a small study of EIB and halted prematurely due to an SAE ultimately deemed unrelated to the study drug (Parker et al., 2011). Recently, a phase 2b study examining multiple doses of MEDI-528 vs placebo in 327 subjects failed to show significant benefit in terms of FEV1, ACQ, or rate of asthma exacerbations (Oh et al., 2012). No subgroups demonstrating enhanced clinical benefit were described, but the lack of a signal in all comers in this study suggests that IL9 is not a critical node in disease pathogenesis and/or that the activity of IL9 was incompletely blocked by MEDI-528. With negative or equivocal results it will be critical to have PD biomarkers data demonstrating pharmacological activity of anti-IL9 for correct interpretation of the role of IL9 is asthma pathophysiology: a lack of efficacy could be the result of incomplete target inhibition and/or lack of a dominant role for IL9 in asthma pathophysiology. The lack of a robust target-related PD biomarker renders distinguishing between these possible explanations difficult.

A posthoc analysis of a large rheumatoid arthritis registry study of the TNFα blocking agent etanercept showed hints of clinical activity in 12 subjects with comorbid asthma (Stoll et al., 2009). Subsequently, a randomized placebo-controlled trial of etanercept in 132 patients with moderate to severe asthma on ICS was conducted. In this study, etanercept was well tolerated but failed to show efficacy on FEV1 or ACQ (Holgate et al., 2011).

In a phase 2 study of golimumab, an mAb against TNFα, in 309 moderate to severe asthmatics, no significant effects were observed for golimumab vs placebo on FEV1 or the rate of severe asthma exacerbations. However, there was a high rate of discontinuations in the active arm due to an increased rate of SAEs, particularly infections, leading to a premature end to the study (Wenzel et al., 2009). Thus, while there is evidence for elevated TNFα in the airways of some asthma patients, TNFα blockade has not shown a favorable risk–benefit profile in asthma.

5. CONCLUSION

Considerable progress has been made in the past 10–15 years toward developing targeted biological therapeutics for asthma. Nevertheless, only one agent (omalizumab) has achieved regulatory approval for the treatment of asthma thus far, and considerable attrition has affected many of the others. An emerging theme from these experiences is that a deeper understanding of human asthma pathophysiology will be necessary to link the biological activities of therapeutic targets with observable clinical outcomes. While preclinical models suggest varying roles for IgE, IL4, IL5, and IL13 in airway obstruction, AHR, mucus metaplasia, infiltration of inflammatory cells, and airway remodeling, it is unclear precisely how these pathophysiological effects translate to outcomes such as FEV1, ACQ, and exacerbations in clinical trials. By the same token, it is as yet unknown whether clinical measurements other than FEV1, ACQ, and exacerbations may more appropriately assess the effects of IgE, IL4, IL5, or IL13 blockade in asthma patients. On the other hand, if end points such as FEV1, ACQ, and exacerbations are the most practical, implementable, and robust clinical outcomes currently available for clinical trial design, a better understanding of which pathophysiological mediators contribute to those outcomes should guide candidate therapeutic target selection. Ultimately, all these considerations must serve the core objective of developing novel therapeutics, which is to demonstrate benefit to patients (Fig. 1.1). Returning to the four reasons discussed in the introduction of this chapter as to why a drug candidate might fail (wrong target, wrong molecule, wrong outcomes, wrong patients), a more optimistic viewpoint might be to consider what we have learned with respect to each of the four components through the extensive efforts toward developing therapeutics for inflammatory targets in asthma and how those lessons may be applied in the future.

5.1. Targets

At least some efficacy in asthma patients has been demonstrated in clinical studies for therapies targeting IgE, IL5, and IL13 (alone or in combination with IL4), while therapies targeting IL4 alone or IL9 have yet to demonstrate convincing efficacy in a randomized placebo-controlled clinical study. Therapies targeting TNFα, while providing some hints at clinical efficacy, exhibit unacceptable safety profiles for asthma patients.

5.2. Molecules

In general, studies of mAbs targeting soluble mediators such as IgE, IL5, and IL13 have shown some efficacy, as have preliminary reports for mAbs intended to deplete specific inflammatory target cells (quilizumab, benralizumab). While nondepleting biologic agents that block cytokine activity by binding to cytokine receptors on target cells have shown PD and clinical activity (pitrakinra and AMG 317), the PK properties of these molecules thus far appear to be less favorable than those of neutralizing anti-soluble mediator mAbs or those targeting circulating cells for depletion.

5.3. Outcomes

Early proof-of-activity studies using bronchial allergen challenge have demonstrated positive outcomes for therapies targeting IgE and IL13 but not for those targeting IL5. In proof-of-concept studies, anti-IL5 therapies failed to show clinical benefit in an unselected population but did show clinical benefit in terms of reducing exacerbations in severe eosinophilic asthma. Anti-IL13 improved lung function in some studies (particularly in selected patient subsets) and has shown trends toward reducing exacerbations but must still be investigated in a study of adequate size and duration powered to demonstrate a clinically meaningful benefit on exacerbations.

5.4. Patients

While targets, molecules, and outcomes are established factors in any drug development program and must be carefully considered in all clinical trials, patient selection has only recently emerged as a key factor that can determine the success or failure of antiinflammatory biologic therapies in asthma. Patients with elevated levels of type 2 cytokine activity and/or eosinophilic airway inflammation identified via biomarkers such as sputum eosinophils, sputum cytokine levels, FeNO, blood eosinophil counts, or serum periostin have demonstrated increased clinical benefit from therapies targeting IL5 or

IL13. It remains to be determined which, if any, of these biomarkers will be most appropriate and robust both for regulatory approval and for widespread clinical use. While in a broad sense the type 2/eosinophilic subtype of asthma, however it is defined, appears to benefit most from therapies targeting these mediators, it is unclear whether more specific subsets of asthma patients with disease driven to a greater or lesser extent by specific targets in the pathway will be identified. In addition, a detailed comparison of techniques, assay platforms, cutoffs, and variability over time of these and any other biomarkers will be necessary to help facilitate personalized therapy. Finally, if any of these emerging therapeutic candidates succeeds in demonstrating clinical benefit in the type 2/eosinophilic subset of asthma, it will define a new unmet medical need: the subset of asthma (which may comprise 50% or more of patients with severe disease) *that does not benefit* from these therapies and that should be the focus of new target discovery efforts.

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ABBREVIATIONS

Ab Antibody
ACC Asthma control composite
ACQ Asthma control questionnaire
AHR Airway hyperreactivity
BAL Bronchoalveolar lavage
BT Bronchial thermoplasty
CBC Complete blood count
CCL CC chemokine ligand
CCR CC chemokine receptor
C_{max} Maximal concentration
EAR Early-phase allergic response
EIB Exercise-induced bronchoconstriction
FDA Food and Drug Administration
FeNO Fractional exhaled nitric oxide
FEV1 Forced expiratory volume in 1 s
ICS Inhaled corticosteroid
Ig Immunoglobulin

IL Interleukin
IND Investigational new drug
iNOS Inducible nitric oxide synthase
IV Intravenous
LABA Long-acting β_2-adrenergic agonist
LAR Late-phase allergic response
LT Leukotriene
LTA Leukotriene receptor antagonist
mAb Monoclonal antibody
MOA Mechanism of action
NIH National Institutes of Health
NKT Natural killer T
NO Nitric oxide
OCS Oral corticosteroid
PD Pharmacodynamic
PEF Peak expiratory flow
PK Pharmacokinetic
PRN Pro re nata (in the circumstances; as needed)
SABA Short-acting β_2-adrenergic agonist
SAE Serious adverse event
SC Subcutaneous
SNP Single nucleotide polymorphism
T2M Type 2 myeloid
Th2 T-helper type 2
TNF Tumor necrosis factor
TSLP Thymic stromal lymphopoietin

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CHAPTER TWO

Protein Therapeutics Targeted at the TNF Superfamily

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Contents

Abstract

Protein-based drugs with their unequivocal specificity achieved the long sought milestone of selectively disrupting cytokine pathways to alleviate ongoing inflammation. Tumor necrosis factor (TNF), a member of the superfamily of cytokines involved in regulating immune and inflammatory processes, provides an exemplary model of protein therapeutics. Antibody and receptor-based inhibitors of TNF modify inflammation leading to dramatic improvement in patients with certain autoimmune diseases. Collectively, the structure, specificity and valence of these protein-based drugs provide direct evidence that the essential mechanism of action is antagonism

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of the ligand–receptor interaction. Accumulating clinical knowledge regarding TNF inhibitors also provide insights into the mechanisms involved in different autoimmune diseases. Experience in the development of an arsenal of biologics directed at TNF has additionally contributed to knowledge toward overcoming the challenges of protein drugs, which include production, delivery, antigenicity and pharmacodynamics. Dramatic clinical outcomes with TNF inhibitors are driving investigation and development of biologics toward other members of the TNF superfamily to selectively alter functional properties of the immune system.

1. INTRODUCTION

Cytokines provide the communication pathways for cells of the immune system in their roles in immune regulation and host defense. The goal of selectively disrupting cytokine pathways to alter ongoing inflammation is being achieved through the unequivocal specificity of several antibody-based drugs. Cytokines function in the extracellular space providing a significant advantage for protein-based therapeutics. However, the discovery and development of proteins for clinical use is a major and expensive process with its own unique issues when compared to those for small molecule inhibitors. Antibodies in particular are difficult to get across the blood–brain barrier. The immunogenicity of protein-based therapeutics also remains a significant concern even with the technical evolution toward fully human antibodies. In this review, we examine the tumor necrosis factor (TNF) superfamily (TNFSF) and the TNF receptor superfamily (TNFRSF) of cytokines as exemplary models for protein therapeutics. New conceptual advances in understanding the immune system and disease pathology have emerged from the results of numerous clinical trials in a variety of autoimmune diseases using biologic-based inhibitors of TNF.

2. THE TNF AND TNF RECEPTOR SUPERFAMILIES

TNF, *aka* TNFα, is a member of a superfamily (TNFSF) of structurally related cytokines that signal through specific cell surface receptors that also form a structurally related superfamily of receptors (TNFRSF) (Bodmer et al., 2002). More than 35 specific ligand–receptor pairs form between the TNFSF and TNFRSF (Bossen et al., 2006). The ligand receptor interactions activate signaling pathways that contribute to many cellular processes including cell growth, survival and apoptosis. These fundamental cellular processes modulated by TNF-related cytokines include inflammation, host defense, and organogenesis of the immune, ectodermal, and

nervous systems (Locksley et al., 2001; Wiens & Glenney, 2011). The defining feature of TNFSF is a TNF homology domain, a β-sheet sandwich that assembles into a functional ligand trimer. Most of the TNFSF members are type II transmembrane proteins that are proteolytically cleaved releasing a soluble trimer from the producing cell. The TNFRSF are type I transmembrane proteins defined by an extracellular cysteine-rich domain. The trimeric structure of the ligand promotes efficient clustering of the specific cell surface receptor, which in turn activates signaling pathways and cellular responses. Bivalent specific antibody to the receptor activates signaling in a similar manner as the native ligand, providing evidence that the clustering mechanism accounts for TNF receptor activation. This feature of the TNFRSF allows specific antibodies to function as surrogate ligands and potential drugs. TNF receptors activate signaling pathways by recruitment of specific adapters to their cytosolic tails following ligand binding. The cytosolic tails of TNFRSF contain either a death domain or TNF-receptor-associated factor (TRAF) recruitment motifs. TNFRSF with death domains recruit and activate caspase proteases involved in apoptosis. The TRAF-interacting motif recruits ubiquitin:E3 ligase complexes, leading to activation of serine kinases, which control NF-κB transcription factors and genes involved in cell survival. Except for the original members, the encoding genes are assigned a numerical designation to the TNFSF or TNFRSF prefix. Several other introductory reviews highlighting the structural architecture and genetics of the TNFSF and TNFRSF molecules are available (Bodmer et al., 2002; Kishore et al., 2004; Locksley et al., 2001).

2.1. Characteristics of TNF as a Drug Target

The structural features of TNF are representative of this superfamily. The gene encoding TNF (*aka* TNFα) is tightly linked to the genes encoding two other TNFSF members, lymphotoxin (LT)-alpha and LT-beta, which together reside within the major histocompatibility complex (MHC) loci on chromosome 6 in humans (Spies et al., 1986). The genes for several other TNFSF members reside in three MHC paralogous regions on Chr19p13, Chr 1q23 and Chr 9q32. The genetic linkage of TNF within the MHC underscores the evolutionarily conserved role that TNF plays in immune responses (Wiens & Glenney, 2011).

The two TNFRs differ in their cellular expression profiles and signaling mechanisms. TNFR1 is widely expressed, whereas TNFR2 is more restricted in expression to hematopoietic and endothelial cells (www.imgen.org). Both TNFR1 and TNFR2 contain similar extracellular domains but signal

through distinct intracellular regions (Schrofelbauer & Hoffmann, 2011; Walczak, 2011). TNFR1 contains a death domain that links TNF binding to apoptotic pathways and is a highly potent activator of NF-κB, leading to induction of many genes involved in acute inflammation (Walczak, 2011). TNFR2 signaling is mediated through TRAF of ubiquitin E3 ligases (Pineda et al., 2007). Studies examining the individual roles of TNFR1 and TNFR2 have identified distinct and separate outcomes, with TNFR1 signaling leading to inflammation and TNFR2 signaling leading to immunoregulation.

Lymphotoxin-α (LTα) is closely related to TNF and shares binding to TNFR1 and TNFR2 but has a very distinct role in immune function from TNF. TNF is connected to a larger network of cell surface and secreted signaling systems through LTα (Ware, 2005). LTα forms a homotrimer (LTα3) that is exclusively secreted, yet it also assembles into a heterotrimer with LT-β where it is anchored to the membrane (Browning et al., 1993). The LTαβ heterotrimer specifically binds a distinct receptor, LTβR (Crowe et al., 1994), which engages another TNFSF member, LIGHT (*TNFSF14*) (Mauri et al., 1998). LIGHT engages herpesvirus entry mediator (HVEM; *TNFRSF14*), a receptor bound also by LTα. The shared ligands and receptors define the immediate TNFSF, which has an even more extended relationship to immunoregulatory pathways through HVEM binding to the Ig superfamily members, B and T lymphocyte attenuator and CD160 (Murphy & Murphy, 2010; Ware & Sedy, 2011).

2.2. TNF Metabolism

TNF is synthesized as a 27-kDa type 2 transmembrane subunit of a trimer that can be shed at the cell surface (17-kDa subunit) into a secreted trimer. The transmembrane form is cleaved to the soluble form by ADAM17, a member of the disintegrin and metalloproteinase family (Saftig & Reiss, 2011; Scheller et al., 2011). Membrane-bound TNF intrinsically enhances the ability of the ligand to cluster its cognate receptors in the target cell significantly enhancing biological potency when compared to the soluble form (Quesniaux et al., 2010; Schneider et al., 1998). This feature is highly relevant to drug targeting as the membrane position can be used to target antibody effector functions to the TNF-producing cell and also influences the compartmentalization and distribution of the drug in vivo.

Many types of cells produce TNF depending on the nature of the stimulus. Activation of Toll-like receptor and other innate sensors by viral,

bacterial and fungal products initiate TNF synthesis in macrophages, mast cells, granulocytes, natural killer (NK) cells, and also in nonhematopoietic cells, such as fibroblasts, neurons, keratinocytes, and smooth muscle cells. In organs involved in immune reactions, such as lymph nodes, gut, epithelium, and synovium, TNF is constitutively transcribed at low levels and contributes to homeostasis of these organs (www.immgen.org). Antigen recognition by T and B cells also initiates TNF production. Lymphocytes and macrophages are likely to be the primary sources of TNF in autoimmune diseases (Li, Hsu, & Mountz, 2012).

TNF biosynthesis is controlled at the transcriptional level. TNF mRNA accumulation is regulated by an AU-rich element in the 3′ mRNA (Caput et al., 1986; Kontoyiannis et al., 1999; Shaw & Kamen, 1986) common to several acute inflammatory cytokines (Seko et al., 2006). TNF expression is also controlled at the level of microRNAs. Pathogen-enhanced phosphorylation of MAPK-activated protein kinase 2, which is critical for maintaining TNF mRNA stability through the microRNA, miR-125b. miR-125b binds to the 3′ untranslated region of TNF mRNA and destabilizes the transcript, whereas miR-155 enhances TNF production by increasing TNF mRNA half-life and limiting expression of SHIP1, a negative regulator of the PI3K/AKT1 pathway (O'Connell et al., 2009; Rajaram et al., 2011; Tili et al., 2007).

2.3. TNF Bioavailability

TNF bioavailability is also controlled by soluble TNFR generated from membrane TNFR by proteolytic cleavage (shedding) (Crowe et al., 1995). Soluble TNFR can be detected in the circulation and levels increase in various disease states and after TNF stimulation (Diez-Ruiz et al., 1995; Pinckard et al., 1997; Xanthoulea et al., 2004).

Activation of lymphocytes and macrophages induces TNF receptor shedding, resulting in acute loss of receptors from the cell surface and temporary desensitization of the cell to TNF binding. Moreover, the soluble TNFR can compete with cellular receptors for free ligand (Xanthoulea et al., 2004). The trimeric structure of TNF dictates that soluble TNFR at low concentration stabilize TNF (only a single site occupied), but at higher concentrations they saturate TNF, blocking all three receptor-binding sites, thus inhibiting activity. This feature allows bivalent forms of soluble TNFR (etanercept) to function only as inhibitors of TNF binding, thus efficiently attenuating TNF activity. The activity and regulation of ADAM17, which cleaves TNFR, and the level of natural ADAM17 inhibitors also contribute

to regulating TNF activity (Freour et al., 2009). Soluble TNFR levels in plasma rise substantially during inflammation (Aderka et al., 1993; Diez-Ruiz et al., 1995).

2.4. Expression of TNF in Human Disease

TNF expression in plasma and other biological fluids is frequently elevated in inflammatory conditions and during infections. In autoimmune diseases such as rheumatoid arthritis (RA), patients had higher levels of TNF compared to healthy subjects, which correlated with key clinical parameters (Beckham et al., 1992). However, the transient nature of TNF expression creates significant variation in TNF protein levels in biological fluids (plasma, synovial, and cerebrospinal fluids) that often does not correlate with disease activity (Fong et al., 1994; Keyszer et al., 1999; Rooney et al., 1995). Alternate markers of TNF signaling with increased dynamic range and stability are being evaluated.

Recent advances in genome-wide assessment of patients with autoimmune and other diseases reveal statistical association with different polymorphic variants in the TNF gene (Table 2.1). Polymorphisms closely linked to TNF and LTα loci associate with a wide range of human diseases including not only multiple sclerosis (MS) and RA but also infectious disease and neurological inflammation (Prajapati et al., 2011). In some cases, previously identified disease associations were not replicated with larger populations and refined statistical analyses. This is not surprising since autoimmune diseases typically show polygeneic characteristics, i.e. weak associations among several genes with no single gene standing out, except for linkage with the MHC. The TNF gene shows strong linkage disequilibrium making it difficult to separate associations from the highly polymorphic variants in the MHC class I genes (Fernando et al., 2008). For example, studies with a much larger patient cohort demonstrated the TNF promoter polymorphism TNF-857 is a risk allele for psoriatic arthritis (PsA) independent of the PSORS1 locus (MHC linked) (Giardina et al., 2011). Detailed understanding of cytokine signaling pathways has provided a mechanism accounting for variants in two genes that act downstream of TNF signaling, regulate NF-κB signaling (*TNIP1*, *TNFAIP3*), and are associated with psoriasis (Ps) (Nair et al., 2009). Most importantly, pharmacogenetic analysis of TNF gene polymorphisms in conjunction with related genes, such as TNFR1 and TNFR2, is providing insight into predicting responsiveness to anti-TNF drugs in patients with Ps (Vasilopoulos et al., 2012).

Table 2.1 Disease associations of polymorphic variants of TNF and LTα genes

	Gene location	Disease	Reference
TNF SNP			
rs1800750	5′ TNF enhancer	Multiple sclerosis	(Favorova et al., 2006)
		Falciparum malaria	(Sinha et al., 2008)
		Behçet disease	(Radouane et al., 2012)
rs361525	Promoter	HIV infection	(Simpson et al., 2012)
rs1799724	5′proximal	Alzheimer disease	(Laws et al., 2005)
rs3093662	5′proximal	Rheumatoid arthritis	(Tang et al., 2009)
LTα SNP			
rs1041981	Coding region 26Thr > Asn	Myocardial infarction	Not involved (Clarke et al., 2006)
rs1041981	Coding region 26Thr > Asn	Ischemic stroke	(Wang et al., 2009)
rs2239704	+80A intron	Leprosy	(Alcais et al., 2007)
rs1041981	Coding region 26Thr > Asn with TNF-308 variant	Psoriatic arthritis	(Balding et al., 2003)

3. ANTIBODY AND RECEPTOR-BASED DRUGS

3.1. TNF Inhibitors

Interest in TNF as a clinical target stemmed from its cytotoxic activity for cancer cells; however, injection of pure TNF induced a shock-like syndrome in humans with cardiovascular and respiratory symptoms associated with systemic inflammation (Selby et al., 1987). The first TNF inhibitors were tested in treatment of bacterial septic shock but proved contraindicated due to overwhelming increase in bacterial sepsis (Fisher et al., 1996). These two "failures," however, provided the clinical insight into the testing of TNF inhibitors in diseases with "sterile inflammation" i.e. autoimmune diseases.

Currently, there are five clinically approved TNF inhibitors for use in treating several different autoimmune diseases (Fig. 2.1). These drugs are all based on the structure of the IgG1 antibody including, infliximab,

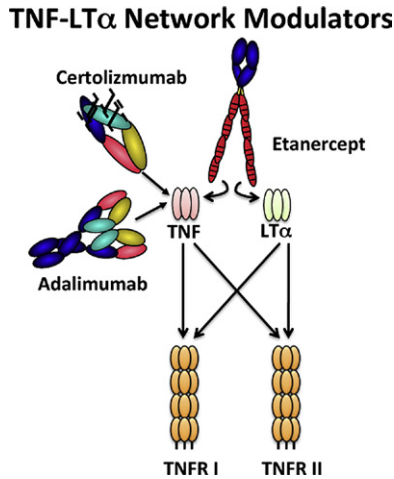

Figure 2.1 ***Inhibitors of the TNF and LTα cytokine system.*** TNF and LTα engage two distinct cell surface receptors, TNFR1 and TNFR2. Both ligands bind both receptors and are cross-competitive (indicated by arrows). Inhibitors in clinical use include antibody-based inhibitors (representative examples include adalimumab and certolizumab). Adalimumab is bivalent fully human monoclonal antibody, whereas certolizumab is a monovalent antibody fragment with enhanced pharmacodynamic properties by chemical addition of polyethylene glycol moieties. In contrast, etanercept is a bivalent fusion protein containing TNFR2 and the Fc region of human IgG1. Etanercept blocks both TNF and LTα from engaging their receptors, whereas the antibody-based inhibitors are specific for TNF and do not act as inhibitors of LTα. (For color version of this figure, the reader is referred to the online version of this book).

adalimumab, certolizumab, golimumab, and etanercept (Table 2.2). The IgG1 class of antibody contains four chains, two heavy (H) and two light (L) chains. Disulfide bonds link H and L and H and H chains together forming a dimer of dimers. The N terminus of each H and L chain contains a variable domain that combines with antigen and the C-terminal domains in the H chain (Fc) provide the effector activity (Kabat, 1988).

All the currently approved TNF inhibitors share a common molecular mechanism of action, which is to competitively inhibit the binding of TNF to its cognate receptors. However, the inhibitors differ in their physical and functional properties, including specificity, valence, effector function, pharmacokinetics and antigenicity. Etanercept is a chimeric fusion of the ectodomain of TNFR2 with the Fc region of IgG1 and thus is specific for both TNF and LTα, whereas the antibody-based inhibitors are specific for TNF. Infliximab is derived from a mouse antihuman TNF but reconstructed with human IgG1 and κ constant regions, whereas adalimumab and golimumab are complete human IgG1 molecules with H and L chains (κ).

Table 2.2 Characteristics of clinically approved TNF inhibitors

	Etanercept	Infliximab	Adalimumab	Certolizumab	Golimumab
Class	Fc fusion protein	Monoclonal antibody	Monoclonal antibody	Monoclonal antibody fragment	Monoclonal antibody
Structure	Hu TNFR2-Fcγ1	Mo/Hu chimeric IgG1κ	Hu IgG1κ	PEG-Hu G1,κ Fab	Hu IgG1κ
MW (kDa)	120	150	150	~95	150
Specificity	TNF/LTα	TNF	TNF	TNF	TNF
TNF ligands	sTNF, tmTNF	sTNF, tmTNF	sTNF, tmTNF	sTNF, tmTNF	sTNF, tmTNF
LT ligands	LTα3, LTα2β1	None	None	None	None

Hu: human; LT: lymphotoxin; Mo: mouse; PEG: polyethylene glycol.
Receptor-based therapeutics denoted by the suffix *cept*, chimeric antibodies are denoted by the suffix *ximab*, humanized antibodies are denoted by the suffix *zumab*, and fully human antibodies by the suffix *umab*.

Certolizumab is anti-TNF derived from mouse complementarity-determining regions spliced into conserved V domains of G1 and κ chains (approximately 95% human origin). Certolizumab in contrast to the other antibody-based drugs is a monovalent Fab fragment (with H and L chains) that has been chemically modified by addition of polyethylene glycol to improve its pharmacokinetic properties (Chen et al., 2011). Interestingly, the fusion protein formed with the TNFR1 (p55/60 (Onercept)) failed in several clinical trials where etanercept is effective, a situation lacking a clear mechanistic basis (Papp, 2010).

3.2. Primary Mechanism of Action

TNF inhibitors act as competitive antagonists preventing soluble and membrane TNF from engaging their receptors (Tracey et al., 2008). Antibody-based TNF inhibitors neutralize TNF by recognizing antigenic epitope in the ectodomain of TNF near or in the receptor-binding region, thus sterically hindering the ability of TNFR to form stable complexes with TNF. One TNF trimer binds three antibody molecules at saturation (Kaymakcalan et al., 2009; Scallon et al., 1995). In contrast, etanercept, the receptor-based therapeutic, binds directly to the receptor-binding region on TNF, which is formed by the interaction of two adjacent subunits in the TNF trimer. Etanercept binds TNF in a 1:2 stoichiometry in either membrane or soluble form. Etanercept also binds soluble LTα with high affinity, and

Table 2.3 Pharmacokinetics of TNF inhibitors

	Etanercept	Infliximab	Adalimumab	Certolizumab	Golimumab
K_D sTNF (pM)★	11	44	127	90	18
K_D mTNF (pM)†	445	468	483	+	+
Half-life ($t½$, days)	4	8–10	10–20	14	7–20

Information collected from the following references.
★Soluble TNF (Nesbitt et al., 2007; Shealy et al., 2010; Tracey et al., 2008).
†Membrane TNF (Kaymakcalan et al., 2009); (+) indicates binding to mTNF (Bourne, Fossati, & Nesbitt, 2008), affinity data not accessible for certolizumab or golimumab.

to a minor form of surface LTαβ, the LTα2β heterotrimer, but not to the major LTα1β2 form. The bivalency of etanercept enhances avidity when compared with natural soluble TNFR (Kaymakcalan et al., 2009). Without TNF binding to its receptor, the signaling cascade resulting in acute inflammation is silenced, thereby blocking the inflammatory responses.

TNF inhibitors differ in their pharmacokinetic profiles (Table 2.3) and may contribute to the different efficacies of these antagonists. Etanercept is rapidly cleared from the serum at 72 ml/h (compared to 11 ml/h for infliximab and 12 ml/h for adalimumab) and has the shortest half-life at 4–5 days. Intact IgG1 has a long half-life in vivo, which is reflected in the longer half lives of infliximab (8–10 days), adalimumab and golimumab. The PEGylation of certolizumab extends the half-life of the Fab to 10–20 days. Etanercept has the lowest maximum steady state concentration of 1.1 μg/ml (compared to 118 μg/ml for infliximab and 4.7 μg/ml for adalimumab). Together, these differences suggest that the anti-TNF antibodies may provide longer coverage than etanercept, which is reflected in the increased dosing schedule for etanercept (Moots & Naisbett-Groet, 2012).

The mechanisms of turnover of TNF inhibitors are characteristically similar to those that metabolize antibodies and immune complexes. Immune complexes are taken up by Fc receptor bearing phagocytic cells of the reticuloendothelial system. Other routes of direct antibody-induced internalization of membrane TNF may occur but are untested in vivo. The pharmacodynamic parameters of the TNF inhibitors are also dependent on clinical variables including demographic variables (e.g. body weight, age and sex), concurrent medications (methotrexate and prednisone, both prolong inhibitor's half-life), inflammatory burden, and immunogenicity.

All these parameters contribute to the variability in concentrations of drug in plasma and in the clinical response (Colombel et al., 2012).

All protein-based drugs, whether of mouse or human origin, generate antidrug antibodies, which can profoundly alter therapeutic usefulness. Antidrug antibodies have been demonstrated with all the approved TNF inhibitors, with the mouse chimeric proteins more profoundly immunogenic than fully human antibodies (Bartelds et al., 2011; Sandborn, 2010). The antidrug antibody response is influenced by several factors including differences in species-specific protein sequences that generate foreign epitopes, the patient's immune status, use of concomitant immunosuppressants, route of administration and dosing regimen (Tabrizi et al., 2006). Circulating antibodies to infliximab from previous treatments can influence levels of certolizumab (Lichtenstein et al., 2010). As antidrug antibodies increase in concentration, the drug levels proportionally decrease with an associated loss in the drug half-life.

The evolutionary function of antibodies is to link antigen recognition with effector mechanisms of the immune system. The membrane position of TNF in cell culture models allows infliximab, adalimumab and golimumab to induce antibody-dependent cytotoxicity (ADCC), activate complement and engage Fc receptors in cells expressing TNF including macrophages and NK cells, thus potentially eliminating inflammatory cells. Binding or cross-linking of membrane TNF is suggested to induce an intracellular signaling cascade (Horiuchi et al., 2010), although such pathways by TNF-related ligands are undefined. Some debate over the role of these secondary mechanisms in the efficacy of TNF inhibitors is reported in the literature (Dinarello, 2005; Nesbitt et al., 2007; Van den Brande et al., 2003). These secondary mechanisms are contributed by the Fc region and bivalency of the antibody-based inhibitors. However, the efficacy of certolizumab as a monovalent and Fc-less molecule provides a strong argument that the primary mode of action of these drugs is by direct antagonism of ligand–receptor binding. However, some of these secondary mechanisms may play a role in infectious disease such as reactivated tuberculosis in treated patients (Fallahi-Sichani et al., 2012; Wallis, 2011).

Second-generation drugs targeting TNF include ESBA105, a TNF inhibitory single-chain human antibody fragment (26 kDa) with potentially better access to some tissues including cartilage and brain (Furrer, Berdugo, et al., 2009, Furrer et al., 2009; Ottiger et al., 2009; Urech et al., 2010). In addition, reengineered TNFR2-Fc with higher affinity for TNF may provide enhanced antiinflammatory activity compared to etanercept

(Edwards, 1999; Yang et al., 2010). Other more-innovative but less-established technologies on the horizon include biologics with dual specificity, such as tandem scFv-Fc, Fab-scFv, and diabodies (Hornig & Farber-Schwarz, 2012).

4. CLINICAL RESPONSES TO TNF INHIBITORS

4.1. Efficacy

The efficacy of TNF inhibition varies among different autoimmune diseases, differences occur in the efficacy among the different TNF inhibitors in a particular disease, and significant heterogeneity is observed in the response of patients with a given autoimmune condition. This variation in drug and patient responses reveals important aspects of human disease (Table 2.4).

4.1.1. Different Autoimmune Diseases

All the approved TNF inhibitors have profound impact in certain autoimmune diseases, including RA and Ps, but not in several other autoimmune conditions including systemic lupus erythematous (SLE) and Sjögren syndrome (SS) or other inflammatory conditions such as osteoarthritis. TNF inhibitors are contraindicated in MS promoting onset or exacerbating disease symptoms. Recent evidence from genome-wide analyses revealed a genetic variant in TNFR1 (rs1800693) that is associated with increased

Table 2.4 Clinical indications for TNF inhibitors

Drug	Commercial name	Indications	Contraindication	No effect
Etanercept	Enbrel	RA, Ps, PsA, AS	MS	IBD, SLE, OA
Infliximab	Remicade	RA, Ps, PsA, AS, IDB	MS	SLE, OA
Adalimumab	Humira	RA, Ps, PsA, AS, IBD	MS	SLE, OA
Golimumab	Simponi	RA, Ps, PsA, AS, IBD	MS	SLE, OA
Certolizumab	Cimzia	RA, Ps, PsA, AS, IBD	MS	SLE, OA

RA, rheumatoid arthritis; Ps, psoriasis; PsA, psoriatic arthritis; AS, ankylosing spondylitis; IBD, inflammatory bowel disease, includes both Crohn disease (CD) and ulcerative colitis (UC); OA, osteoarthritis; MS, multiple sclerosis.

risk for MS. This genetic variation results in synthesis of a soluble form of TNFR1 (not by shedding), thus higher levels of soluble TNFR appear to mimic the effect of added TNF inhibitors in MS (Gregory et al., 2012). The clinical reality of all TNF inhibitors is their use is in conjunction with other disease-modifying small molecule drugs, such as the antimetabolite drug, methotrexate, or the antiinflammatory drug, prednisone (Bathon et al., 2000). Several newer small molecule inhibitors are in clinical trials (Fleischmann, 2012).

4.1.2. Heterogeneity of Response in a Given Autoimmune Condition

About two-thirds of patients with RA show a strong or partial response to inhibition with any of the TNF inhibitors (Feldmann & Maini, 2010), yet the remaining patients show no significant response. The basis of this failure to respond is not well understood but suggests that TNF-independent mechanisms drive the disease-causing inflammation.

All currently available anti-TNF inhibitors are effective in treating symptoms associated with RA and related arthritides including juvenile idiopathic arthritis with improvement in symptoms (joint stiffness and pain), physical ability (movement) and slower progression of joint deterioration (radiographic evidence) (Chen et al., 2006; Kay et al., 2008; Keystone et al., 2008). Patients with RA and related inflammatory arthropathies have premature stiffening and thickening of the arterial walls. Patients treated (>1 year) with anti-TNF inhibitors had improvement in aortic stiffness and slowing down of progression in the carotid intima media thickness (Angel et al., 2012), demonstrating long-term positive outcome.

Patients with active and progressive Ps and PsA show improvement of both skin and joint symptoms with etanercept or the antibody-based inhibitors (Boyce et al., 2010; Fleischmann, 2010; Kay & Rahman, 2010; Kerensky et al., 2012; Keystone et al., 2008; Mease, 2006; Murdaca et al., 2011).

Some patients initially respond to therapy but fail over time. Antidrug antibodies are believed to be the primary cause; however, other unknowns may also contribute (Ritchlin, 2010). In some cases, increasing dose or switching inhibitors is reported to be effective. For example, adalimumab has also been proved to be effective in patients with moderate to severe Ps who failed to respond to etanercept (Martyn-Simmons et al., 2009). Additional approaches in patients who fail one TNF inhibitor include combination with newer cytokine inhibitors (Alwawi et al., 2009; Menter, 2009). However, the combination of anti-TNF and interleukin (IL)-1 blockade

(anakinra) in patients with RA showed significant increase in infections. This lesson suggests the need for careful preclinical research and clinical analysis on combination therapy.

4.1.3. Disparity in Efficacy of TNF Inhibitors

Inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn disease (CD), revealed significant differences in the efficacy of TNF inhibitors, with etanercept showing no impact in IBD (Sandborn & Targan, 2002). Etanercept is also not efficacious in treating sarcoidosis, Wegener granulomatosis, or uveitis (Haraoui, 2005) in contrast to infliximab, which shows efficacy in treating these rare inflammatory disorders (Atzeni et al., 2007; Brik et al., 2007; Keystone, 2004). Ligand specificity is the most striking difference between etanercept and the antibody-based drugs; etanercept engages both LTα and TNF. Little is known about LTα in IDB and other diseases, but the ability of LTα to engage inhibitory signaling via HVEM-BTLA may make its case as a Cinderella cytokine.

The remission induced by anti-TNF in CD is remarkable, closing fistulas and significantly reducing hospitalization and surgery (Lichtenstein et al., 2010, Lichtenstein et al., 2005; Talley et al., 2011). Although infliximab is highly effective in treating CD, some patients become infliximab resistant and intolerant to further administration due to antidrug antibodies and induction of serum-sickness-like syndrome. Switching from infliximab to the fully human adalimumab can provide efficacy and is well tolerated without signs of immunogenicity (Papadakis et al., 2005). Increased dosage may also overcome resistance (Billioud et al., 2011).

4.2. Off Label Investigations

Numerous off-label studies with TNF inhibitors in patients with systemic autoimmune conditions have been reported with variable results. Anti-TNF inhibitors (infliximab and adalimumab) show promise in treating ocular inflammation in Behçet disease, a multisystem inflammatory disorder of unknown etiology (Benitah et al., 2011). Some case reports indicate a therapeutic response to infliximab in sarcoidosis; however, continued investigations indicate that there is insufficient evidence to ensure the efficacy of TNF antagonists in sarcoidosis (Maneiro et al., 2012). Results from controlled trials showed lack of efficacy for the use of infliximab in SS and etanercept in SS and Wegener granulomatosis (Ramos-Casals et al., 2008).

The use of infliximab in inflammatory myopathies and sarcoidosis and etanercept in polymyositis were limited due to excess of side effects (>50% of reported cases).

4.3. Pharmacogenetics

Large-scale genetic analysis is revealing key gene associations with responsiveness and side effects to TNF inhibitors (Prajapati et al., 2011). For example, the RA risk allele (protein tyrosine phosphatase, CD45, rs10919563) is associated with the response to anti-TNF therapy, with stronger association in patients with autoantibodies (rheumatoid factor and anti-citrullinated protein) (Cui et al., 2010). Genetic variations in the TNF and LTα loci may contribute to increased susceptibility to upper respiratory tract infections and urinary tract infections in patients treated with etanercept (Hughes et al., 2004).

There is no recognized off-target effect of TNF inhibitors; however, there are significant but rare side effects attributed to the mechanism of action. The on-target side effects include increased risk of serious infection, lymphoma, neurologic (demyelinating), hematologic (pancytopenia) and immunologic (autoantibody) symptoms. Risk of infection is not unexpected given the role of TNF in host defense mechanisms (Wallis, 2011). The most profound risk is increased susceptibility or reactivation of latent disease with *Mycobacterium tuberculosis*. In animal models (Ehlers et al., 2003; Kindler et al., 1989; Roach et al., 2001) and in humans (Fallahi-Sichani et al., 2012) *M. tuberculosis*, infections can become persistent with formation of granulomas that effectively wall off the organism. TNF and related cytokines are essential to organize cells into a granuloma, and thus blockade of TNF results in dissolution of the granuloma and release of bacteria. However, TNF inhibition did not increase risk in all infectious agents. A review of herpes zoster infection in a large cohort of RA patients receiving TNF inhibitors (Strangfeld et al., 2009) revealed an occurrence of 86 episodes in 5040 patients. Although reaching statistical significance for antibody-based TNF inhibitors, the results did not reach threshold for clinical significance. Older patients with autoimmune conditions and under treatment with TNF inhibitors did not have increased risk for adverse infection to live viral vaccine (herpes zoster vaccine, live attenuated) (Zhang et al., 2012), which provides an option to avert recurrence of herpes zoster in the patient population.

TNF inhibitors are contraindicated in patients with MS, and TNF inhibition is associated with induction of MS such as demyelinating disorders

(Solomon et al., 2011). The link between TNF as a key host defense signaling system and the onset of MS suggests that in these rare cases, a pathogen may be escaping control due to TNF inhibition. This idea is supported to a first approximation from the efficacy of the potent antiviral cytokine interferon-β used to treat MS patients.

The success of TNF blockade of inflammation in certain autoimmune diseases has spurred investigations into many other indications. A survey of clinical trials targeting TNF revealed 76 ongoing trials (clinicaltrials.gov). Most of these investigate more detailed aspects of the effect in autoimmune diseases and also include studies examining the impact in cancer and infectious diseases including grade I acute graft-versus-host disease (GVHD), treatment of pulmonary dysfunction after allogeneic stem cell transplant and pemphigus vulgaris.

5. TARGETING OTHER TNFSF PATHWAYS

A disease in which TNF inhibition has little or no effect and in conditions in which a significant subgroup of patients do not respond suggests that other mechanisms of inflammation and immune responses may communicate pathogenesis. Targeting other members of the TNFSF and TNFRSF has provided proof of principle that other cytokine pathways may be involved in pathogenesis (Table 2.6). For example, belimumab, an antibody directed to BAFF (TNFSF13b), was approved in SLE but prescribed for a subset of SLE patients who exhibit B-cell dysfunction as measured by anti-nuclear antibodies (Furie et al., 2011; Navarra et al., 2011). Moreover, within this subset of patients 40–60% failed to significantly respond to belimumab (Stohl & Hilbert, 2012; Treml et al., 2009). Another approved antagonist antibody against TNFSF member 11, RANK ligand, denosumab, inhibits osteoclast survival limiting bone resorption (Body et al., 2006). Denosumab is used in postmenopausal women with osteoporosis at high risk for fractures, in cancer patients to increase bone mass to prevent fractures caused by androgen deprivation therapy (nonmetastatic prostate cancer), in breast cancer patients receiving adjuvant aromatase inhibitor therapy, and in the prevention of skeletal-related events in patients with bone metastases from solid tumors (Christenson et al., 2012; Peddi et al., 2012; Spencer et al., 2012). Anti-CD40L showed promise in autoimmune disease, but the reagent induced coagulopathy, unexpectedly revealing CD40L as a platelet-expressed protein. A newer version of anti-CD40L designed not to

Table 2.5 Human disease linkage in the HVEM network

Gene	Disease association	References
TNFSF14 (LIGHT)	Linked to *IBD6*	(Cheung, Coppieters, et al., 2010; Tello-Ruiz et al., 2006)
	Multiple sclerosis	(Sawcer et al., 2011)
TNFRSF14 (HVEM)	Multiple sclerosis	(Sawcer et al., 2011)
	Celiac disease	(Dubois et al., 2010)
	Rheumatoid arthritis	(Coenen et al., 2009; Kurreeman et al., 2012)
	Sclerosing cholangitis	(Folseraas et al., 2012)
	Follicular lymphoma	(Cheung, Johnson, et al., 2010)
	Obesity	(Bassols, Moreno, Ortega, Ricart, & Fernandez-Real, 2010)
	Ulcerative colitis	(Anderson et al., 2011)
BTLA	Breast cancer	(Fu et al., 2010)
*LT*β	Sjögren syndrome	(Bolstad et al., 2012)

induce coagulopathy is in clinical development. Antagonists to several other TNFSF members including TL1A, OX40, 41BB, and TWEAK are in clinical development.

TNF is a component in a larger network of cytokines defined by shared ligand and receptor usage (Ware, 2005). HVEM (*TNFRSF14*) pathways are involved in both proinflammatory and inhibitory signaling (Murphy & Murphy, 2010; Ware & Sedy, 2011). Large-scale genetic linkage studies have revealed that polymorphic variants of HVEM are associated with a variety of diseases (Table 2.5). Interest in these molecules as clinical targets has been spurred by results that antagonists (decoy receptors and antibodies) to LTαβ and LIGHT alter inflammatory processes in several disease models in mice (Browning, 2008; Steinberg et al., 2011). Particularly interesting is the role of HVEM pathways in chronic inflammatory models (Albring et al., 2010; Doherty et al., 2011; Soroosh et al., 2011). Agonizing inhibitory pathways via BTLA may offer additional routes to modulating immunity in disease pathogenesis (Murphy & Murphy, 2010; Pasero et al., 2009; Steinberg et al., 2011).

The complexity of the TNF-LT-LIGHT network in activating proinflammatory and inhibitory pathways begs the issue of designing a biologic

that arrests inflammatory signaling while preserving or agonizing the inhibitory pathway. Current antagonists in clinical development or clinical trials include decoy of the LTβR, anti-LTα momoclonal antibody (mAb), and anti-LIGHT mAb. A soluble Ig decoy receptor of LTβR (baminercept/BG9924) is a competitive inhibitor of both LIGHT and LTαβ, analogous in structure to etanercept. Baminercept engages soluble and cell surface forms of these ligands but affects the inhibitory signaling from HVEM-BTLA/CD160 interactions. Baminercept showed a safe profile in a phase I trial and entered a phase II trial in RA. This manufacturer-sponsored trial was curtailed due to the inability to achieve an ACR-50 end point within 3 months. However, further analysis revealed significant movement in selected biomarkers and with clinical improvement in a subset of patients. Baminercept is currently in a phase II trial for Sjögren syndrome now sponsored by the NIH. Antibodies to LTα or LIGHT are also in clinical development for autoimmune disease. A humanized antibody to LTα (pateclizumab/MLTA3698A) possessing ADCC activity was used to deplete T cells in a xenogenic GVHD model with disease amelioration (Chiang et al., 2012), and a phase I study in RA showed good safety with mild/moderate adverse events (Emu et al., 2012). Previous data in mouse models indicated that helper type1 cells display surface LTαβ and that elimination of these effectors via depleting antibody decreased tissue damage in neurological and joint disease (Chiang et al., 2009). A second phase I trial is planned to compare pateclizumab with adalimumab in RA. A fully human antibody to LIGHT (SAR252067) competitively inhibits LIGHT binding to all of its three receptors, HVEM, LTβR and DcR3. Evidence in animal models indicates that targeting the LIGHT network in IBD may be an appropriate clinical indication (Browning, 2008; Cohavy et al., 2005; Doherty et al., 2011; Gatumu et al., 2009; Shaikh et al., 2001; Ware, 2009). The complexity of the larger TNF/LT/LIGHT network provides substantial room for creative approaches to drug development. Here, lessons learned from pathogens may provide key insights into drug design (Sedy et al., 2008).

6. CONCLUSION

TNF inhibitors have revealed new insights into human immune and inflammatory systems and the mechanisms involved in disease processes. This accumulated knowledge is allowing new insights into the processes of inflammation in human disease mechanisms. For example, TNF blockade revealed TNF as an important mediator of inflammation in RA, Ps

and IBD. Likewise, we appreciate that autoimmune diseases can be distinguished at the molecular level based on their sensitivity to blockade by TNF and eventually with other cytokine pathways. A significant aspect of stopping treatment with TNF inhibitors is that the pathogenic process remerges; hence the disease is not cured. The implication that homeostasis is not restored by TNF blockade alone raises the issue of what other pathways might be involved in reestablishing homeostasis? Is it possible to restore homeostasis through combined modulators with other cytokine pathways in conjunction with TNF inhibitors? Recent evidence demonstrates that therapy with adalimumab, but not etanercept, induces T regulatory cells that restrain IL-17-associated inflammation in RA via regulation of monocyte-derived IL-6 (McGovern et al., 2012; Nadkarni et al., 2007). New drugs added on to TNF inhibitors should be coming online in the near future to address such key issues. Of course, the decisions to include combination therapy will require firm scientific basis but will also need to satisfy multiple socioeconomic factors (Table 2.6).

Table 2.6 TNF superfamily systems in clinical development

TNF/TNFRSF	Name	Biologic	Clinical indication
BAFF	Benlysta™	Human IgG antagonist	Lupus (approved)
	Tabalumab	Human IgG antagonist	Autoimmune disease
BAFFR/TACI	Atacicept	Fc chimeric of TACI-Fc	Autoimmune disease
CD27L	SGN-75	Anti-CD27L-drug conjugate★	Cancer
	MDX-1411	Human IgG1, Fc modified	Cancer
	MDX-1203	Anti-CD27L drug conjugate†	Cancer
CD27	CDX-1127	Human anti-CD27 Agonist	Cancer
CD30	Adcetris™	Antibody drug conjugate‡	Hodgkin lymphoma (approved)
	XmAb2513	Humanized anti-CD30, Fc modified	Cancer (HL, ALCL)
	MDX-1401	Human anti-CD30, Fc modified	Cancer

Continued

Table 2.6 TNF superfamily systems in clinical development—cont'd

TNF/TNFRSF	Name	Biologic	Clinical indication
CD40	ASKP1240	Antagonist mAb	Transplantation
	Lucatumumab	Depleting mAb	Cancer
	Dacetuzumab	Depleting IgG1 agonist function	Cancer
	Chi Lob 7/4	Depleting IgG1 agonist function	Cancer
	CP-870,893	Agonist IgG	Cancer
41BB	BMS-663513	Human anti-4-1BB IgG agonist	Cancer
	PF-05082566	Human anti-4-1BB IgG2	Cancer
GITR	TRX518	Agonist IgG, Fc modified	Cancer
LTα	MLTA3698A	Depleting IgG1 antibody	Autoimmunity
LTβR	Baminercept	Decoy-Fc	Autoimmunity
LIGHT	SAR252067	Human anti-LIGHT	IBD
OX40L	Oxelumab	Human anti-OX40L IgG1	Asthma
OX40	AgonOX	Mouse anti-OX40	Cancer
TRAILR1	Mapatumumab	Human agonist IgG	Cancer
TRAILR2	Conatumumab	Human agonist IgG	Cancer
	Drozitumab	Human agonist IgG	Cancer
	Lexatumumab	Humanized agonist IgG	Cancer
TWEAK	BIIB023	Antagonist humanized mAb	Autoimmune disease
TWEAKR	PDL192	Agonist	Cancer
	RO5458640	Agonist	Cancer

The drugs listed here as of September 2012 are in clinical development or trials that can be searched at clinicaltrials.gov.

⋆Anti-CD27L (CD70) conjugated with monomethyl auristatin phenylalanine (MMAF).

†Human anti-CD27L (CD70) conjugated with duocarmycin using a cathepsin B protease release mechanism.

‡Chimeric anti-CD30-conjugated to MMAE (monomethyl auristatin E).

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ABBREVIATIONS

AS Ankylosing spondylitis
CD Crohn disease
IBD Inflammatory bowel disease
LTα Lymphotoxin
MS Multiple sclerosis
OA Osteoarthritis
Ps Psoriasis
PsA Psoriatic arthritis
RA Rheumatoid arthritis
UC Ulcerative colitis

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CHAPTER THREE

Immunopharmacology of Lipid A Mimetics

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Contents

Abstract

The structural core of bacterial lipopolysaccharide, lipid A, has played a role in medicine since the 1890s when William Coley sought to harness its immunostimulatory properties in the form of a crude bacterial extract. Recent decades have brought remarkable clarity to the structure of lipid A and the multicomponent endotoxin receptor system

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that evolved to detect it. A range of therapeutically useful versions of lipid A now exists, including preparations of detoxified lipid A, synthetic copies of naturally occurring biological intermediates such as lipid IVa, and synthetic mimetics. These agents are finding use as vaccine adjuvants, antagonists and immunostimulants whose structural features have been refined to potentiate efficacy while decreasing the risk of inflammatory side effects.

1. INTRODUCTION

The existence of cellular receptors for recognizing "pathogen-associated molecular patterns" as a component of adaptive immune priming was first postulated by Janeway (1989). Janeway believed that by recognizing conserved patterns on infectious microbes, these pathogen recognition receptors provided a mechanism for rapid detection and response to infection that would then augment and direct the slower but more discriminating adaptive response. This hypothesis was dramatically confirmed with the discovery of mammalian Toll-like receptors (TLRs) (Lemaitre et al., 1996; Medzhitov et al., 1997; Taguchi et al., 1996) and identification of the lipopolysaccharide (LPS) receptor, TLR4, in 1998 (Poltorak et al., 1998).

Ten mammalian TLRs have been characterized and recognize conserved microbial molecular targets including bacterial lipopeptides (recognized by TLR2/1 and TLR2/6 heterodimers), CpG DNA (TLR9), viral RNA (TLR3, TLR7, and TLR8), bacterial flagellin (TLR5), and LPS (TLR4). TLRs are primarily found on cells in tissues and on mucosal surfaces where microbial invasion can be detected early and a robust response mounted. TLR activation stimulates rapid production of inflammatory mediators and recruitment of cells from the systemic innate defenses. Production of inflammatory mediators and activation of TLRs on antigen-presenting cells (APCs), including dendritic cells, then provide signals for induction of the more specific and longer lasting acquired immune response to resolve the primary infection and protect against repeat infections.

In addition to providing a greater understanding of the functional relationship between innate and adaptive immunity, study of TLRs has revealed the importance of these receptors in the efficacy of many vaccines and immunotherapeutics. However, the potent and potentially dangerous inflammatory and pyrogenic response induced by TLR agonists such as LPS often prevents their clinical use. Therefore, there is growing interest in the design and development of safe TLR agonists for use as innate and adaptive immunomodulators (Dunne et al., 2011; Duthie et al., 2011; Johnson, 2008). For instance, substantial resources have been applied to the development

of synthetic LPS analogs in an effort to reduce the immunopathology while improving the therapeutic activity (Hawkins et al., 2002; Johnson, 2008; Tamai et al., 2003). This chapter will describe the progress made in the design of TLR4-based therapeutics and recent insights into the interplay of structure and biology for their activity.

2. LPS RECEPTOR COMPLEX

2.1. LPS-binding protein

A cascade of binding proteins, including LPS-binding protein (LBP), CD14, and the heterodimeric MD-2/TLR4 complex, sequentially contributes to the biological recognition and response to LPS (Fig. 3.1). The reason for the existence of this complex, four-component cascade of sequential

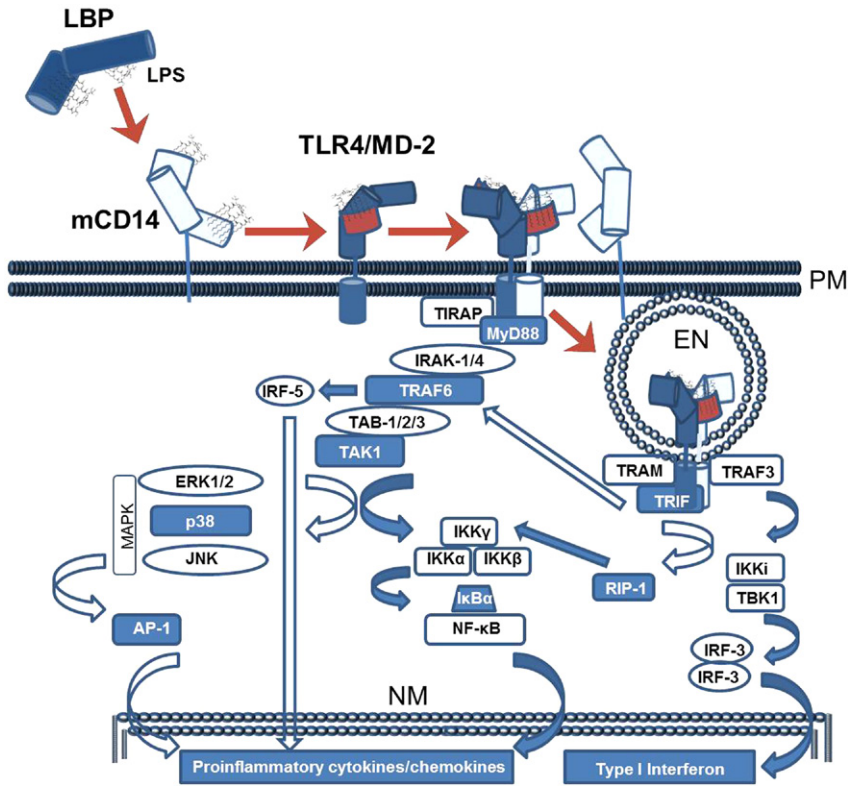

Figure 3.1 MyD88- and TRIF-dependent pathway signaling downstream of TLR4 as described in the text. PM, plasma membrane; NM, nuclear membrane; EN, endosome. (For color version of this figure, the reader is referred to the online version of this book).

interactions may be to balance the requirement for extreme sensitivity in detection of microbial infections with the necessity for exquisite regulation of crucial but potentially dangerous inflammatory outcomes (Gioannini & Weiss, 2007). Earliest in the sequence, LBP extracts LPS from bacterial membranes or free aggregates and transfers monomers to soluble or membrane-bound CD14 (Schumann et al., 1990; Wright et al., 1990).

LBP is a secretory acute-phase protein primarily synthesized in liver hepatocytes in response to bacterial infection (Grube et al., 1994; Schumann et al., 1996) and belongs to the lipid transfer/LBP family of proteins that includes bactericidal/permeability-increasing protein (BPI) and phospholipid transfer protein (PLTP). The crystal structure of LBP has not been determined (Schumann, 2011), but the structure of the closely related BPI suggests that hydrophobic pockets on opposite sides of the boomerang-shaped structure may be responsible for binding the acyl chains of ligands such as LPS (Beamer et al., 1997; Beamer et al., 1998).

LBP is not only important for early detection of serum LPS during bacterial infection, but production of high serum levels of LBP during infection and sepsis helps sequester LPS in order to dampen the detrimental inflammatory effects that manifest in this systemic disease (Lamping et al., 1998; Zweigner et al., 2001). LBP reduces the risk of septic shock by extracting LPS from bacterial membranes or membrane blebs and transferring it directly to plasma lipoproteins such as high-density lipoprotein (HDL) (Mathison & Ulevitch, 1979; Munford et al., 1981). Lipoprotein-bound LPS is inactivated as an endotoxin and is eventually cleared from circulation in the liver (Pajkrt et al., 1996; Parker et al., 1995). The essential position of LBP as the sentinel serum protein in the detection of bacterial LPS suggests that it may provide an ideal target of inhibition for prevention of septic response; however, the secondary role of LPB in mop-up of LPS during acute infection makes therapies to inhibit its function more risky.

2.2. CD14

CD14 is a member of the family of leucine-rich repeat (LRR) proteins with widely disparate functions that also include the TLRs (Botos, Segal, & Davies, 2011). CD14 is expressed primarily by myeloid cells as a glycosylphosphatidylinositol (GPI)-linked membrane or soluble glycoprotein (Gegner et al., 1995; Jerala, 2007), but is also present in soluble form in serum (Kielian & Blecha, 1995). Transfer of LPS monomers from LBP to CD14 is catalytic (Hailman et al., 1994), with what is believed to be a

horseshoe-shaped dimer of CD14 binding two molecules of LPS in N-terminal hydrophobic cavities, surrounded by a rim of cationic residues (Kim et al., 2005). Both soluble (sCD14) and membrane CD14 (mCD14) can transfer LPS to a complex of TLR4 and MD-2 on cells (Gioannini & Weiss, 2007; Kielian & Blecha, 1995); however, mCD14 is 10–100 times more active in transducing a response to LPS (Heumann et al., 1992). sCD14 has also been shown to have a role in activation of some cell types that express TLR4 and MD-2 but lack mCD14 (Kielian & Blecha, 1995).

Shortly after the discovery of CD14, it was suspected that mCD14 might be the lone high-affinity receptor for LPS on cells (Wright et al., 1990). However, even before the discovery of TLR4, researchers proposed the existence of additional interacting receptors (Ulevitch, 1993). The lack of a transmembrane signaling domain on CD14 and the fact that many cells lacking CD14 could respond to LPS stimulation, albeit at high concentrations of agonist (Lee et al., 1992), suggested that other receptors or coreceptors might be involved. Those coreceptors later proved to be TLR4 and MD-2.

Recently, CD14 has been tied to more diverse functions than merely increasing the potency of the response to LPS. For instance, it has also been proposed that CD14 regulates dimerization of TLR4/MD-2 heterocomplexes (Tsukamoto et al., 2010) and regulates TLR4/MD-2 receptor endocytosis in response to free or bacterial LPS (Zanoni et al., 2011). In addition, CD14 appears to regulate the ability of the TLR4/MD-2 complex to signal through the signaling adaptor Toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-β (TRIF)-dependent signaling pathway downstream of TLR4 (Jiang et al., 2005; Zanoni et al., 2011), probably due to the localization of TRIF-mediated signaling to the endosomal membrane (Kagan et al., 2008; Tanimura et al., 2008).

The presence of CD14 has also shown to be necessary for the TLR4/MD-2 complex to respond to smooth chemotypes of LPS (S-LPS) with a complete core oligosaccharide and O-polysaccharide chain (Jiang et al., 2005). In these studies, the TLR4/MD-2 complex responded only to the rough LPS (R-LPS) chemotype, which lacks the O-polysaccharide, or to the lipid A molecule, which lacks all saccharides, in the absence of CD14. This activity might be ascribed to a chaperon function of CD14, facilitating otherwise less favorable interactions between the S-LPS molecule and the receptor complex, as is reflected in the ability of CD14 to bind and transfer such divergent molecules as endotoxin, phospholipids and lipopeptides (Nilsen et al., 2008; Yu et al., 1997).

R-LPS and lipid A signaling in the absence of CD14 was limited almost exclusively to the signaling adaptor myeloid differentiation response gene 88 (MyD88)-dependent pathway downstream of TLR4, and CD14 was essential for TRIF-dependent signaling in response to either S-LPS or R-LPS. However, the presence of CD14 marginally increased the potency of MyD88-dependent signaling outcomes as well (Zanoni et al., 2011). The authors speculated that CD14 is an essential factor that allows the LPS receptor to switch into a "full activation" mode (MyD88 plus TRIF-dependent signaling) in response to S-LPS or R-LPS by inducing a supramolecular (i.e. multimerization of receptor complexes on the cell surface) or conformational change permitting TRIF-dependent signaling (Jiang et al., 2005). It is tempting to speculate that CD14 itself could serve to catalyze receptor dimerization as the distance between the LPS binding pockets on CD14 (Kim et al., 2005) corresponds remarkably closely with the distance between LPS molecules in the crystal structure of a dimer of two TLR4/MD-2/LPS complexes (Park et al., 2009).

The diversity of activities regulated by CD14 suggests that the coreceptor may be a good target for therapeutic intervention, either for inhibition of hyperactivation during sepsis or for adjuvanting of innate responses to weakly immunogenic antigens. However, the relatively indiscriminate recognition of endotoxins and lipopeptides by CD14 and the additional influence of CD14 on TLR2 signaling may make therapeutic targeting a challenge. However, Peri et al. (2007) have successfully inhibited CD14 activities in inflammation and sepsis with synthetic, dialkyl amino glycolipids and benzylammonium lipids, and more recently with a symmetric lipid A mimetic formed by two dialkyl glucose units linked through a 6–6′ succinic diamide linker (D1) (Piazza et al., 2012). Amino glycolipids and benzylammonium lipids inhibited activation of cells with LPS by binding CD14 and preventing transfer of LPS to cell surface TLR4/MD-2 (Piazza, Rossini, et al., 2009; Piazza, Yu, et al., 2009). D1 inhibited TLR4 activation by competing with endotoxin for binding both CD14 and MD-2 (TLR4 coreceptor). However, D1 not only inhibited endotoxin activity but was itself a weak TLR4 agonist, suggesting it may be promising as a potential vaccine adjuvant as well (Piazza et al., 2012).

2.3. TLR4/D-2

Poltorak et al. (1998) used positional cloning to prove that the mutations that caused the hyporesponsiveness of C3H/HEj and C57Bl/10ScCr mice

to LPS resided in the *Tlr4* gene. TLR4 is a 95 kD type 1 integral membrane protein with an extracellular LRR domain that functions in ligand recognition and a cytoplasmic TIR domain that functions in signal transduction (Kim et al., 2007). Interestingly, however, LPS-unresponsive cells are not made responsive with TLR4 expression alone (Kirschning et al., 1998; Shimazu et al., 1999), leading some to postulate mistakenly that TLR2 was the LPS receptor (Kirschning et al., 1998; Yang et al., 1998), probably due to contamination of LPS preparations with lipopeptides that activate TLR2 (Hirschfeld et al., 2000). Shortly thereafter, a search for homologs to MD-1, a coreceptor that binds the TLR receptor homolog RP105 expressed on lymphocytes, led to the discovery of the true TLR4 coreceptor, MD-2 (Shimazu et al., 1999).

MD-2 is a 25 kD secreted protein that exists primarily in stable complex with the ectodomain of TLR4 on the surface of cells. MD-2 interacts directly with TLR4 but also provides the primary interaction surface for bound endotoxin molecules (Gioannini & Weiss, 2007; Viriyakosol et al., 2001; Visintin et al., 2005), while TLR4 functions in signal transduction (Visintin et al., 2005). The β-cup structure of MD-2 includes a hydrophobic pocket large enough to accommodate up to five acyl chains of a typical lipid A molecule (Kim et al., 2007; Ohto et al., 2007; Park et al., 2009).

A comparison of the crystal structures of MD-2 with bound Eritoran or lipid IVa, two LPS antagonists for the human receptor that both have only four acyl chains (Kim et al., 2007; Ohto et al., 2007), with the crystal structure of a dimer of two TLR4/MD-2/LPS complexes (Park et al., 2009) suggested some structural determinants for agonist/antagonist activity.

First, although all four acyl chains of the antagonists pack tightly in the hydrophobic pocket of MD-2, only five acyl chains from LPS could be accommodated. A sixth acyl chain attached through C2 of the reducing glucosamine of LPS is forced outside of the pocket and forms the core of a hydrophobic interface between the two heterocomplexes (TLR4/MD-2/LPS), stabilizing dimerization. Second, accommodation of a fifth acyl chain in the MD-2 pocket displaces the glucosamine backbone of LPS upward ~5 Å relative to the antagonist complexes, exposing the 1- and 4′-phosphates and hydrophilic ester and amide groups connecting the acyl chains to interact with charged residues on MD-2 and on both TLR4 molecules (Park et al., 2009). It appears to be that the additional hydrophobic and electrostatic interactions made possible by this structural rearrangement provide the necessary binding energy to promote

dimerization of two TLR4/MD-2/agonist complexes and signaling through the receptor.

3. TLR4 SIGNALING

Signaling to the TLR4/MD-2 complex in vivo begins with the extraction of LPS from bacterial membranes or aggregates by LBP and subsequent transfer to soluble or membrane CD14 (Fig. 3.1) When CD14 in turn transfers the LPS to the TLR4/MD-2 complex on the cell surface, binding of two molecules of LPS induces dimerization and activation of two major downstream signaling pathways, the MyD88- and TRIF-dependent signaling pathways (Park et al., 2009; Saitoh et al., 2004; Stover et al., 2004; Visintin et al., 2003). Upon dimerization, two TIR domain-containing adaptor proteins (TIRAPs), Mal/TIRAP and MyD88, bind the complex through the cytoplasmic TIR domains of TLR4. MyD88 recruits the IL-1 receptor-associated kinase (IRAK)-4 and IRAK-1 or IRAK-2 to a forming "Mydossome" through interactions with their death domains (Flannery & Bowie, 2010). Phosphorylation and activation of the IRAKs begin a signaling cascade that results in early activation of MAP kinases (p38, ERK1/2, c-Jun N-terminal protein (JNK)) and NF-κB. The MAP kinases activate the transcription factor AP-1, which cooperates with NF-κB to induce expression of proinflammatory cytokines in the nucleus (Checker et al., 2012).

Endocytosis of the TLR4/MD-2 receptor complex initiates the TRIF-dependent phase of signaling (Kagan et al., 2008). TRIF binds to the endosomal complex through interaction with the TRIF-related adaptor molecule (TRAM/TICAM-2) (Rowe et al., 2006; Yamamoto et al., 2003). Signaling through the TRIF-dependent pathway results in delayed NF-κB activation that is lower in amplitude than MyD88 and occurs through an alternative pathway involving receptor-interacting protein 1 (RIP1) (Cusson-Hermance et al., 2005; Meylan et al., 2004; Yamamoto et al., 2003). Also, downstream of TRIF-dependent signaling are the interferon regulatory factors (IRF)-3 and IRF-7 (Kawai et al., 2001; Yamamoto et al., 2003) that regulate transcription of type I interferons (IFNα/β) and expression of receptors (CD86, CD80) for T-cell costimulation (Hoebe et al., 2003). Later, autocrine or paracrine IFN signaling induces IFN-inducible chemokines such as IFN-inducible protein-10 (IP-10) and regulated and normal T cell expressed and secreted

(RANTES) (Kawai et al., 2001; Serbina et al., 2003; Teghanemt et al., 2005; Yamamoto et al., 2003b).

4. ENDOTOXIN

Richard Pfeiffer was the first to describe an "endotoxin" constituent of Gram-negative bacteria in 1892 (Westphal et al., 1981). This heat-stable component of the bacterial membrane he characterized is now known as LPS, the major component and structural feature of the outer leaflet of the outer cell membrane of Gram-negative bacteria. LPS is best known as the causative agent of endotoxic or septic shock, the systemic inflammatory syndrome characterized by hypotension, fever and dysregulated coagulation that can lead to organ failure and death in extreme cases (Salomao et al., 2012; Van Amersfoort et al., 2003). However, low levels of endotoxin exposure can have therapeutic effects. In fact, clinicians and scientists have exploited the immunomodulatory effects of endotoxins for over 100 years. In the 1890s, William Coley unwittingly used endotoxin as a component of his "Coley's Toxins" in treatments for bone sarcomas (Oblak & Jerala, 2011). Vaccinologists have been utilizing endotoxin and other TLR ligands as part of the "dirty little secret" in formulations of effective Gram-negative bacterial vaccines since the late 1800s with little understanding of the molecular basis for the enhanced effect (Arakawa, 2011; Janeway, 1989).

The potential for extreme toxicity generally precludes LPS-like molecules from consideration in therapeutic or prophylactic applications. However, the efficacy of LPS formulations in preventing or limiting cancer progression has intrigued clinicians since the time of William Coley to delve further into its potential therapeutic uses (Lundin & Checkoway, 2009). Therefore, subsequent research has focused on strategies to harness its beneficial immune-stimulating properties while avoiding the toxic side effects. Later, with the characterization of LPS structure described below, researchers began testing the efficacy of natural LPS isolates and other derivatives in cancer models, some with striking preclinical results (Jeannin et al., 1991; Mizushima et al., 1999). Unfortunately, it has become clear that many obstacles to safe and effective endotoxin-based therapies still need to be overcome, including intolerable side effects and the induction of tolerance, a state in which the TLR4/MD-2 endotoxin receptor system becomes temporarily refractory to further stimulation (Jeannin et al., 1991; Mizushima et al., 1999; Oblak & Jerala, 2011).

5. STRUCTURE OF LPS

Early attempts at crude purification of endotoxin using trichloroacetic acid (TCA) or organic solvents indicated a composition of carbohydrate and lipid with little if any associated protein (Luederitz et al., 1965). Westphal and Luderitz developed techniques to further purify endotoxin from enteric bacteria using hot phenol–water extraction, a method that yielded a product they termed LPS that was composed of lipid, carbohydrate and phosphorus components (Caroff et al., 2002; Freudenberg & Galanos, 1990; Luederitz et al., 1965; Rietschel et al., 1994; Sutherland et al., 1965). The complete structure of LPS was finally determined in the 1960s based on the work of such pioneers as Westphal, Luederitz, Rietschel and Galanos (Caroff et al., 2002; Freudenberg & Galanos, 1990; Rietschel et al., 1994).

LPS structure varies considerably between bacterial species but generally consists of three principal structural domains: the outermost O-polysaccharide or O-antigen chain, the core oligosaccharide and the lipid A moiety that anchors the molecule in the outer membrane (Caroff et al., 2002; Osborn et al., 1964) (Fig. 3.2). The O-polysaccharide consists of a highly variable polymer of up to 50 repeating sugars and is the main contributor to the antigenic specificity of the "O-antigen" antibacterial response (Erridge et al., 2002). The core oligosaccharide is divided into an outer core of 5–10 hexoses proximal to the O-chain and an inner core of 2–3 heptose sugars attached to 1–3 residues of 3-deoxy-D-mannooctulosonic acid (KDO) (Caroff et al., 2002; Erridge et al., 2002; Osborn et al., 1964). The inner-most domain, referred to as lipid A, consists of a commonly diphosphorylated glucosamine disaccharide. The glucosamine residues frequently have 3–4 N- or O-linked β-hydroxy acyl chains of 10–16 carbons often with 2–3 additional acyl chains attached through the β-hydroxyl groups (Frirdich & Whitfield, 2005; Heinrichs et al., 1998). Lipid A is also crucial for maintaining the structural integrity of the outer membrane and is the most highly conserved domain (Rietschel et al., 1984; Seydel et al., 1984).

6. LIPID A: THE "ENDOTOXIC PRINCIPLE"

Boivin and colleagues were the first to identify lipid A as the toxic component of LPS. They extracted LPS with TCA and hydrolyzed the product in acetic acid at 100 °C to produce a lipid precipitate they named "fraction A," that retained the toxic effects of the parent molecule (cited

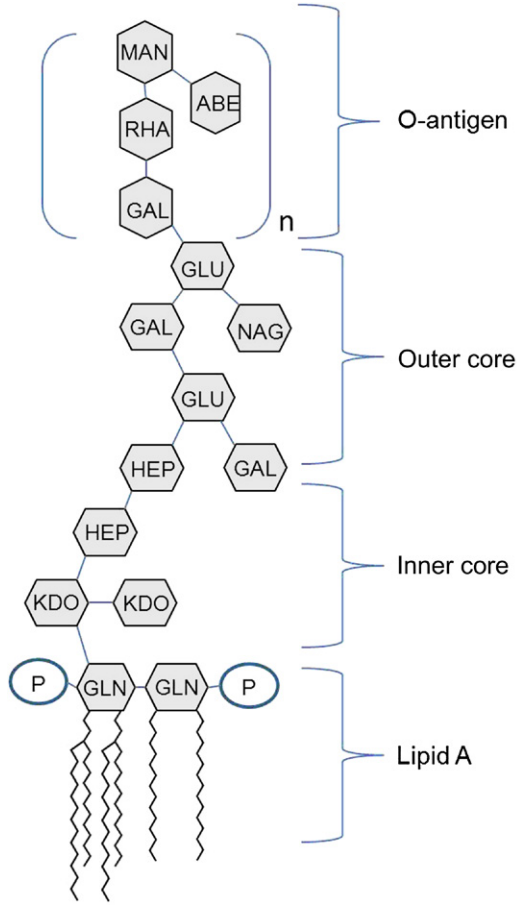

Figure 3.2 General structure of Gram-negative LPS as described in the text. P, phosphate (PO_3^-); MAN, mannose; ABE, arabinose; RHA, rhamnose; GLU, glucose; NAG, N-acetyl glucosamine; HEP, heptose; KDO, 3-deoxy-D-mannooctulosonic acid; GAL, galactose; GLN, glucosamine.

in Nowotny, 1987). In attempting to elucidate the structure of LPS, Luderitz et al. extracted LPS with hot phenol and found that lipid A could be easily separated from the carbohydrate domains by subsequent hydrolysis with HCL at 100 °C to cleave the ketosidic linkage (Rietschel et al., 1982; Rietschel, Zahringer, et al., 1984). Comparisons of the activity of free lipid A molecules (solubilized with serum albumin) with LPS in biological tests, such as galactosamine-sensitized mouse lethality, rabbit pyrogenicity and complement inactivation, identified lipid A as the domain most likely

responsible for the endotoxic effects, and lipid A was designated accordingly the "endotoxic principle" (Luderitz et al., 1973).

Lipid A's importance for toxicity was later confirmed with the creation and testing of synthetic preparations of lipid A, both partial and complete structures, based upon the natural products from *Escherichia coli* and *Salmonella minnesota* (Galanos et al., 1971; Rietschel et al., 1971). In fact, a synthetic lipid A corresponding to the predominant species found in preparations from *E. coli* (compound 506, Fig. 3.2) had strikingly similar in vivo and in vitro biological activities to natural preparations (Kotani et al., 1985). Therefore, the β(1→6)-D-glucosamine disaccharide carrying phosphates at positions 1 and 4′ and (R)-3-hydroxytetradecanoyl groups in positions 2, 3, 2′ and 3′ with additional 3-hydroxydodecanoic and 3-hydroxytetradecanoic fatty acids at positions 2′ and 3′ (Fig. 3.2) was deemed the minimal structure for optimal lipid A activity (Rietschel et al., 1987). Further analysis of this series of synthetics revealed that those lacking one or both phosphates and with fewer (4–5) or more (Kotani et al., 1983) acyl chains were less active in at least one of the various biological activities tested: mouse lethal toxicity, rabbit pyrogenicity, local Schwartzman reactivity, Limulus gelation, tumor necrotizing activity, B cell mitogenicity or induction of prostaglandin (PG) in macrophage cultures. Elucidation of the determinants of lipid A activity opened the floodgates for exploration of structure–activity relationships by chemical synthesis of numerous modified species.

7. DIVERSITY OF LIPID A IN NATURE

Although lipid A is the most conserved domain of Gram-negative bacterial LPS, the molecule still shows a great degree of diversity among bacterial species. Differences abound in the number and modifications of the phosphate residues, the number and length of the acyl chains and, though less common, the chemistry of the disaccharide backbone (Trent et al., 2006). Although the lipid A biosynthetic pathway is highly conserved in Gram-negative bacteria, it also has an adaptive component (Raetz et al., 2007). A conserved pathway includes nine constitutive enzymes that are either intracellular or are integral inner membrane proteins with active sites that face the cytoplasm and produces an *E. coli*-type diphosphoryl, hexa-acylated diglucosamine-KDO_2 lipid A. This is the predominant structure found in Gram-negative bacteria with only limited variability between species, usually in the length or spatial configuration of the acyl chains (Raetz & Whitfield, 2002). From here, bacteria can adapt to temperature, adjust to

growth conditions or respond to the threat of antimicrobial peptides or membrane-disrupting polymyxins from Gram-positive bacteria by expression of lipid A modifying enzymes in the periplasmic space and outer membrane. These enzymes are responsible for adding core and O-polysaccharide chains, for modifying the 1- and 4′- phosphates (removing phosphates or modifying phosphates with 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine, or methyl groups) and for adding or removing acyl chains (Bishop, 2005; Raetz et al., 2007; Trent, 2004).

Many pathogenic bacteria, including *Yersinia pestis*, *Francisella tularensis*, *Helicobacter pylori*, *Legionella pneumophila*, *Porphyromonas gingivalis* and *Pseudomonas aeruginosa*, modify lipid A structure to help avoid recognition by host defense mechanisms, particularly the CD14/TLR4/MD-2 complex (Albers et al., 2007; Cigana et al., 2009; Curtis et al., 2011; Dixon & Darveau, 2005; Hajjar et al., 2006; Miller et al., 2005; Moran, 2010). These modifications are often responsive to host conditions and produce structures that diverge significantly from the "canonical" structures conserved in enteric bacteria like *E. coli* (1- and 4′-diphosphoryl, diglucosamine with six acyl chains from 12 to 14 carbons in length (Fig. 3.3)) and are often less immunogenic (Loppnow et al., 1989; Takada & Kotani, 1989). For example, *Y. pestis* can change the composition of lipid A from a mixture of tri-, tetra-, penta-, and hexa-acylated species to a mixture of primarily tri- and tetra-acylated lipid A species (Fig. 3.4a) in response to a temperature shift from the temperature of its flea vector (27 °C) to the temperature of the human host (37 °C) (Kawahara et al., 2002; Rebeil et al., 2004). This modification substantially reduces activation of the TLR4 receptor complex and is likely an adaptation to avoid innate immune response by the mammalian host (Matsuura et al., 2010; Robinson et al., 2008; Telepnev et al., 2009).

In a manner similar to *Yersinia* spp., *P. gingivalis*, an important agent of periodontal disease, appears to alter its lipid A structure in response to conditions in the oral cavity. At relatively high haemin concentrations, mimicking conditions of vascular ulceration during the disease state, *P. gingivalis* produces a monophosphorylated, tetra-acylated lipid A. This structure is an immunosuppressive and TLR4 antagonistic form for human cells (Coats et al., 2009; Zhang et al., 2008). However, at relatively low haemin concentrations, *P. gingivalis* produces a nonphosphorylated, tetra-acylated and a monophosphorylated, penta-acylated lipid A, both relatively weak TLR4 agonist (Al-Qutub et al., 2006).

Bacteroides thetaiotaomicron, an important intestinal symbiont in humans, is phylogenetically closely related with *P. gingivalis*. *Bacteroides thetaiotaomicron*

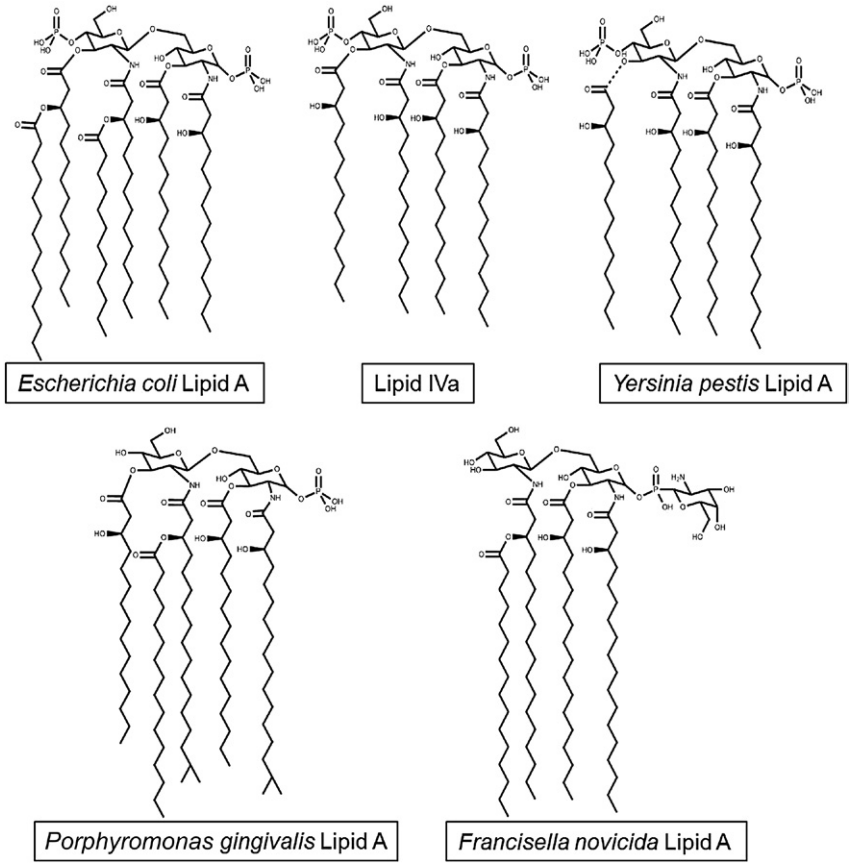

Figure 3.3 Lipid A structures isolated from *E. coli* and bacterial pathogens.

produces a monophosphorylated and penta-acylated LPS that is structurally very similar to that of *P. gingivalis* (Fig. 3.3) (Berezow et al., 2009). However, the penta-acylated *B. thetaiotaomicron* LPS has a 4′-phosphate and is significantly more immunostimulatory than the penta-acylated LPS of *P. gingivalis* with a 1-phosphate (Coats et al., 2009, 2011). The authors postulated that genetic regulation of the position of the monophosphate on the LPS species affects their immunogenicity and contributes to the choice of niche of the bacteria as commensals or pathogens.

Legionella pneumophila, *Francisella* spp. and *P. aeruginosa* also produce poorly immunogenic lipid As with only four or five acyl chains of varying carbon lengths (Miller et al., 2005; Trent et al., 2006). These lipid A species are either weak agonists or even antagonists for the TLR4/MD-2 receptor complex on host immune cells. Therefore, conditional manipulation of the

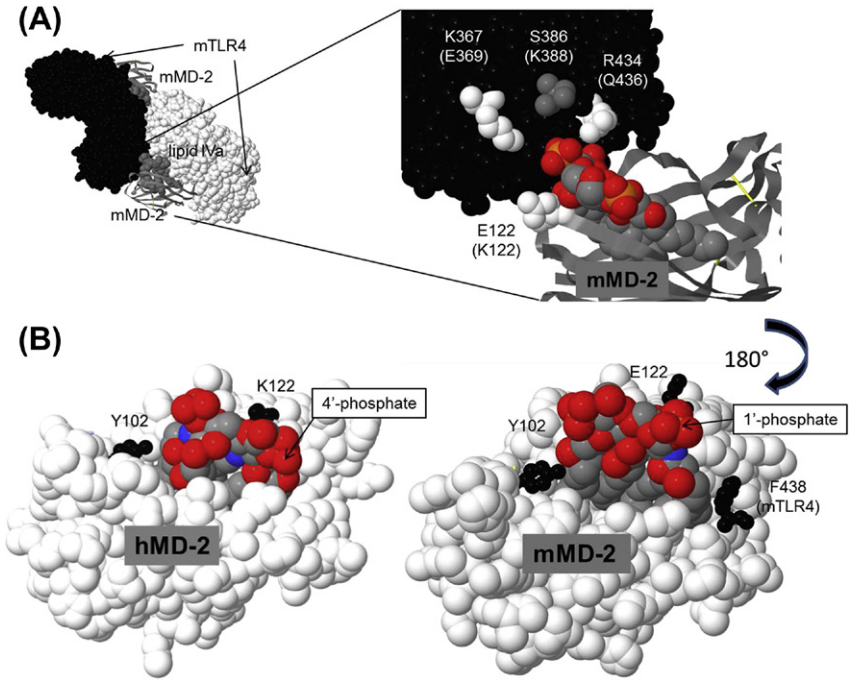

Figure 3.4 Structure of lipid IVa as an agonist in complex with a murine TLR4/MD-2 (mTLR4/mMD-2) dimer and as an antagonist in complex with human MD-2 (hMD-2). (A) mTLR4/mMD-2/lipid IVa dimer complex and detail of ionic interactions at the dimerization interface. (B) Comparison of lipid IVa complex with hMD-2 and mMD-2. Examples of MD-2 residues interacting with lipid IVa are shown in black along with a residue (F438) from the partner mTLR4 shown in a hydrophobic interaction with C3–C7 of the acyl chain at the 2 position of lipid IVa (Fig. 3.3). For color version of this figure, the reader is referred to the online version of this book.

number and/or length of acyl chains on the lipid A species, and to a lesser extent, the degree of phosphorylation of lipid A, seems to be a common strategy of Gram-negative bacterial pathogens to evade detection by the innate immune system and contributes significantly to their virulence.

As outlined above, poorly immunogenic LPS can prevent a potent innate immune response to pathogenic bacteria, resulting in low initial bacterial clearance and, potentially, a less effective subsequent adaptive response. The importance of the LPS-dependent innate response for resistance to infection was recently illustrated. Airhart et al. showed that mice treated with a potent synthetic TLR4 agonist 24 h before infection with a normally lethal inoculum of *Y. pestis* showed dramatically decreased bacterial burden in the lungs, prolonged time-to-death and significantly improved survival relative

to controls. Protection correlated positively with cytokine induction and was TLR4-dependent (Airhart et al., 2008).

The same strategy was effective against infection with *Francisella novicida*, a murine infection modeling human infection with *F. tularensis*, the causative agent of pneumonic tularemia or "rabbit fever" (Lembo et al., 2008). *Francisella tularensis* and *F. novicida* predominantly express a tetra-acylated and monophosphorylated LPS at 37 °C to avoid detection by the host innate response (Fig. 3.3) (Hajjar et al., 2006; Kanistanon et al., 2012). Mice treated with a relatively potent synthetic TLR4 agonist before inhalation of *F. novicida* showed reduced bacterial replication in the lung, spleen and liver, prolonged time-to-death and increased survival. As evidence that adaptive immunity was established as well, survivors were protected from subsequent reinfection by virulent *F. novicida* (Lembo et al., 2008).

8. SYNTHETIC LIPID A

Synthesis of lipid A analogs began with the desire to understand the structure of the domain of LPS principally responsible for the endotoxic activity seen in sepsis. Two developments, in particular, were critical for elucidation of this "endotoxic principle." First, the lipid A component of LPS was isolated and characterized by the group of Westphal et al. in Freiburg, Germany (Rietschel et al., 1973; Rietschel et al., 1987). Second, a series of lipid A analogs was synthesized by Shiba et al. in Osaka, Japan based on the structures proposed from chemical analysis of natural lipid A. These analogs were then compared to natural lipid A and LPS for biological activity in close collaboration with the groups in Freiburg, and later Borstel, in Germany (Kotani et al., 1985; Rietschel et al., 1987; Rietschel et al., 1994).

The heterogeneity of preparations of LPS and even lipid A from natural sources made chemical analysis of the endotoxic principle difficult. Consequently, the first series of analogs synthesized were based on what turned out to be a misinterpreted structure of lipid A from *S. minnesota* LPS. These analogs showed very poor activity relative to natural lipid A, prompting some to question the absolute importance of lipid A in endotoxicity (Kotani et al., 1983).

Due to the apparent discrepancies in activity, the Osaka group carefully reanalyzed the chemical structure of the lipid A of an *E. coli* 08:K27 Re mutant, focusing on determination of the location of acyl groups on the diglucosamine backbone. This time, they found evidence for the presence of ester-linked acyl chains on C-3 and C-3′ and amide-linked acyl

chains on C-2 and C-2′ of the diglucosamine. Surprisingly, they did not detect acyl chains attached through the hydroxyl groups at C-4 and C-6′, indicating they could possibly be linkage sites for the polysaccharide chain instead (Takada & Kotani, 1989). This analysis led to a new proposal for the structure of lipid A from *E. coli* (Fig. 3.3) (Shiba et al., 1984), followed by elucidation of the related structures of lipid A from *Salmonella typhimurium* and *S. minnesota* (Fig. 3.3) (Kasai et al., 1986).

With the correct structure in hand, the Osaka group embarked on synthesis of a new series based on that structure and, in collaboration with the groups in Freiburg and Borstel, found that the synthetic version (compound 506, Fig. 3.3) had nearly identical endotoxic activities to the isolated natural lipid A (Galanos et al., 1985; Kotani et al., 1985).

8.1. *Escherichia coli* Lipid A Analogs

Following the synthesis and biological verification of the structure of *E. coli* lipid A by the Shiba group in Osaka in 1985 (Kotani et al., 1985), the group and their collaborators in Germany set about to analyze the structural determinants for in vivo and in vitro biological activity of a series of synthetic analogs differing in the number of acyl chains and the number of phosphate residues. Exhaustive analysis of modified lipid As and partial structures in the series led the authors to the following conclusions (Takada & Kotani, 1989). (1) The number of acyl chains and phosphate residues was important for activity in classic endotoxin tests including chick embryo lethality (CEL), rabbit pyrogenicity and preparation of a thrombohemorrhagic (Schwartzmann) reaction. Analogs with six acyl chains similar to *E. coli* lipid A (Fig. 3.3) were more responsive than those with seven, five or four in order of decreasing activity, while compounds with fewer than four acyl chains or monosaccharide compounds were virtually inactive. Analogs with two (4′- and 1-bisphosphates) phosphate residues were more active than those with one (4′- or 1-monophosphates), which were, in turn, much more active than dephosphorylated compounds with the same number of acyl chains. (2) The degree of acylation was less important than the degree of phosphorylation for toxicity in tests utilizing galactosamine-sensitized mice. Compounds with four, five or seven acyl chains were almost as active as similar analogs with six, while the activity of diphosphoryl compounds was much greater than monophosphoryl and dephosphorylated compounds, which were scarcely lethal. (3) The induction of several inflammatory cytokines associated with the endotoxic response (TNFα, IFNα/β, and PGE2) (Salomao et al., 2012) in mice or cell cultures was dependent on both the

degree of acylation (6>7,5,4) and phosphorylation (2>1>0) of the analogs, while the induction of a primary mediator of the febrile response, IL-1α/β, was only modestly dependent on either acylation or phosphorylation in murine or human cell cultures.

Based on the data from the Osaka group, it appears that with the exception of IL-1α/β induction, most activities associated with the endotoxic response are dependent upon the degree of acylation and phosphorylation of the lipid A analogs. Those analogs with six acyl chains were consistently the most endotoxic, while those with more (7) or fewer (5,4,3) were substantially less toxic. Analogs with two phosphates were more endotoxic than those with one or zero phosphates and the same number of acyl chains. Toxicity in galactosamine-treated mice was not sensitive to the degree of acylation; however, this was possibly due to the dependence of this test on acute liver toxicity, which turned out not to be directly related to symptoms normally associated with endotoxic shock (Mignon et al., 1999).

The Osaka group's series of synthetic lipid A analogs was also tested for vaccine adjuvant activity in several systems. However, the structural requirements for adjuvanticity of the compounds were much less strict than those for endotoxicity. For instance, compounds with from four to six acyl chains induced similar antibody responses to either sheep red blood cells (SRBC) or bovine serum albumin (BSA) coadministered in mice (Takada & Kotani, 1989). The number of acyl chains also did not have a large impact on induction of B-lymphocyte proliferation in in vitro stimulation assays. However, increasing the degree of phosphorylation from zero to one (1- or 4′-monophosphate) to two (1,4′-diphosphoryl) had a small effect on the activity in these assays.

Overall, it appeared from the comparisons of the Osaka group's synthetic lipid A analogs that inflammatory or endotoxic activity of the compounds generally increased from four to six acyl chains and then decreased again with more than six, while the activities generally increased with increasing degree of phosphorylation at the 1 and 4′ positions of the glucosamines. By contrast, analogs with four to six acyl chains were remarkably similar in their abilities to adjuvant adaptive responses and stimulate lymphocyte proliferation. Increasing the number of phosphate residues only marginally increased these responses. This was the first indication in the literature that the structural determinants for the harmful inflammatory and endotoxic effects of lipid A analogs might be separable from its beneficial immunostimulatory effects for adaptive responses.

8.2. Lipid IVa

Along with structure–activity relationship studies on synthetic *E. coli* lipid A and partial structures, some of the most instructive studies on the structural determinants of lipid A activity have utilized synthetic and natural antagonists. One molecule that has been a particularly useful tool for functional studies of the LPS receptor is lipid IVa. Lipid IVa is a naturally occurring lipid A precursor from the constitutive biosynthesis pathway and was isolated from a conditional mutant of *S. typhimurium* defective in *3-Deoxy-D-manno* octulosonic acid (KDO) synthesis (Rick, Fung, Ho, & Osborn, 1977). The molecule is the product of the first six biosynthetic enzymes in the pathway, acting primarily in the cytoplasm, on the inner leaflet or as integral inner-membrane proteins (Raetz et al., 2007). Lipid IVa consists of the diphosphoryl, glucosamine disaccharide with two ester-linked and two amide-linked β-hydroxytetradecanoyl acyl chains and lacks the 3-hydoxy secondary acyl chains (SACs), KDO residues, and 4-amino-4-deoxy-arabinose and phosphorylethanolamine modification of the phosphates of natural *S. typhimurium* lipid A (Fig. 3.3) (Rick & Osborn, 1977). The lipid A synthesis group in Osaka produced and characterized the synthetic version of *S. typhimurium* lipid IVa (Takada et al., 1985), that, as it turned out, was the same structure as the *E. coli* lipid A precursor.

The Osaka group found that synthetic lipid IVa had significant activity relative to natural lipid A and identical activity to the natural precursor molecule in all endotoxin activity tests, including rabbit pyrogenicity, CEL, galactosamine-sensitized mouse toxicity and preparative activity for the local thrombohemorrhagic (Schwartzmann) reaction (Takada et al., 1985). The authors also found that similar to the natural precursor, the synthetic version stimulated human serum complement activity and migration of human polymorphonuclear (PMN) cells. Subsequent studies also found lipid IVa to be a potent mitogenic agent in mouse splenic cultures and a potent inducer of inflammatory cytokine expression relative to natural LPS in murine macrophages (Kotani et al., 1985, 1986). Later, more extensive testing of synthetic lipid IVa, however, suggested that lipid IVa not only lacked agonist activity, but was a potent antagonist of LPS activity on human cells. For example, lipid IVa was shown to inhibit the induction of inflammatory cytokines including TNFα, IL-1α/β, and IL-6 upon cotreatment of human peripheral blood mononuclear cells (PBMCs) and monocytes with lipid A or LPS (Feist et al., 1989; Flad, 1990; Flad et al., 1989; Wang et al., 1991).

Despite these interesting but somewhat conflicting results, little was understood about the cellular or molecular basis for the difference in activities of lipid IVa for human and murine cells until the discovery and characterization of the TLR4/MD-2 receptor complex starting in 1998 (Poltorak et al., 1998; Shimazu et al., 1999). For instance, the specificity for antagonism of human TLR4/MD-2 was confirmed when it was shown that lipid IVa was an agonist for Ba/F3 cells, a murine pro-B cell line, expressing murine TLR4/MD-2, but acted as an antagonist on Ba/F3 cells expressing human TLR4/MD-2 (Akashi et al., 2001). In the same study, the authors tested the activity of lipid IVa on Ba/F3 expressing chimeric mouse and human TLR4/MD-2 combinations, and they identified MD-2 as the coreceptor most responsible for the species-specific response to lipid IVa.

The murine TLR4/MD-2 receptor complex is widely recognized as being less discriminating in recognition of lipid A modifications that differ from the "canonical" diphosphoryl, hexa-acylated structure than the human complex (Akashi et al., 2001; Muroi et al., 2002; Steeghs et al., 2008). In addition to lipid IVa and hexa-acylated lipid A, murine TLR4/MD-2 can respond to tetra-acylated lipid A from *Y. pestis* (Matsuura et al., 2010) to penta-acylated lipid A from *P. aeruginosa* (Hajjar et al., 2002), *Neisseria meningitides* (Steeghs et al., 2008), and *Rhizobium sin-1* (Vasan et al., 2007), and to hepta-acylated lipid A from *Salmonella* spp. (Gaekwad et al., 2010; Tanamoto & Azumi, 2000). However, in the same studies, these agonists were poor agonists and/or relatively potent antagonists for the activity of *E. coli* lipid A or LPS on human TLR4/MD-2.

A review of lipid A data suggests that the length and number of fatty acyl chains on lipid A are the principle discriminating factors for human TLR4/MD-2, and hexa-acylated lipid As with acyl chains of 12–14 carbons are the most potent agonists. As MD-2 contains the hydrophobic binding pocket for the acyl chains of lipid A species (Kennedy et al., 2004; Ohto et al., 2007), MD-2 might be the sole discriminator for species specificity within the receptor complex. However, mutagenesis and crystallographic evidence have recently shed light on the roles of both TLR4 and MD-2 in species-specific ligand discrimination.

The earlier thorough characterization, widespread availability and existence of a synthetic version of lipid IVa made this molecule an easy choice for functional studies of the newly discovered TLR4/MD-2 receptor complex. Studies with mixed mouse–human or chimeric coreceptors and mutagenesis of coreceptors alternately suggested that MD-2 (Akashi et al., 2001; Hajjar et al., 2002; Muroi et al., 2002), TLR4 (Lien et al., 2000;

Poltorak et al., 2000) or, more recently, both coreceptors (Meng et al., 2010; Resman et al., 2009) were responsible for the species-specific activity of lipid IVa. These contradictions would later be explained in part by the effects of lipid IVa on the dimerization of the receptor complex. In a manner similar to the homologous IL-1 receptor from which it derives its name, the cytoplasmic TIR domain of TLR4 requires homotypic dimerization for activation and downstream signaling (Medzhitov et al., 1997; Ozinsky et al., 2000). Saitoh et al. (2004) were the first to show that lipid IVa efficiently induced dimerization of the murine, but not the human TLR4/MD-2 receptor complex.

Subsequent mutagenesis data from several groups (Meng et al., 2010; Meng, Lien, et al, 2010; Meng et al., 2011; Muroi & Tanamoto, 2006; Resman et al., 2009; Vasl et al., 2009) indicated the importance of ionic and hydrophobic interactions on both TLR4 and MD-2 for lipid IVa activity on the murine coreceptor. These results led the authors to independently hypothesize that differences in the ionic interactions of the lipid IVa 4′- and 1-phosphates, as well as hydrophobic interactions of acyl chains with MD-2 and TLR4 at the dimer interface of the TLR4/MD-2 coreceptor, govern the efficiency of dimerization and, therefore, activation of signaling by lipid IVa. Remarkably, interchanging only a handful of amino acids, E122 on MD-2, and K367, S386 and R434 on TLR4 of the murine complex with the corresponding human counterparts (K122, E369, K388, Q436) made lipid IVa an antagonist, while changing the human K125, K122, R69 and Y42 on MD-2 and E369 and Q436 on TLR4 to the murine counterparts (L125, E122, F69, G42, K367, R434) allowed lipid IVa to activate the coreceptor for signaling (Meng, Drolet, et al., 2010).

One prediction from these studies was that ionic interactions between the phosphate(s) of lipid IVa and MD-2 residues with the charged groups on TLR4 (K367/R434) at the dimer interface, interactions that are lacking on the human receptor complex, could provide the energy to stabilize dimerization and signaling (Fig. 3.4) (Meng, Drolet, et al., 2010; Meng et al., 2011). Another prediction from related studies was that differences in the hydrophobic interactions of the lipid IVa acyl chains with specific hydrophobic residues within the murine and human MD-2 binding pocket would force lipid IVa to adopt more of an agonist conformation in the murine complex, exposing hydrophobic acyl chain residues to interact with the partner TLR4 at the dimerization interface (Resman et al., 2009; Teghanemt et al., 2008). Many of the interactions at the lipid IVa/MD-2/TLR4 interface predicted to determine the species specificity of lipid

IVa activity by the mutagenesis studies were dramatically born out with the publication of the crystal structures of human MD-2 with Lipid IVa (Ohto et al., 2007), followed by the structure of a dimer of the human TLR4/MD-2/LPS complex (Park et al., 2009), and, finally, the publication of structures of murine TLR4/MD-2/lipid IVa along with the dimer of murine TLR4/MD-2/LPS (Ohto et al., 2012).

A comparison of the human and murine structures revealed that the diglucosamine backbone of lipid IVa is shifted upward 4–7 Å relative to MD-2 and its orientation is reversed in the antagonist (human) complex relative to the agonist (murine) complex (Fig. 3.4). The 1′-phosphate of lipid IVa faces the monomer TLR4 in the human complex, while the molecule is flipped 180° in the MD-2 pocket and the 1′-phosphate faces TLR4 at the dimer interface in the murine complex (Fig. 3.4b) (Ohto et al., 2012). As predicted, the orientation of the 1′-phosphate in the murine complex is stabilized in the crystal structure by interactions with K367 and R434 on TLR4 at the dimer interface (Meng, Lien, et al, 2010; Meng et al., 2011), although the 4′-phosphate was initially predicted to be in this position (Meng et al., 2011; Resman et al., 2009). Interestingly, E122 of murine MD-2 is also important for stabilizing the lipid IVa orientation. In this case, the repulsion of the 1-phosphate with this negatively charged residue appears to favor an active conformation, while K122 of human MD-2 appears to interact with both 4′- and 1-phosphates and glucosamine backbone in the antagonist structure, favoring deeper binding in the hydrophobic pocket of MD-2 and preventing interactions that would stabilize the dimer interface in an active complex (Ohto et al., 2012).

Many of the predictions from the mutagenesis studies for the differences in hydrophobic interactions of the agonist and antagonist structures were also confirmed by the crystal structures. For instance, it was predicted that in the agonist conformation, an acyl chain from lipid IVa would protrude outside the hydrophobic pocket of MD-2 and form part of a hydrophobic interaction surface between MD-2, lipid IVa and the dimer partner TLR4 (Resman et al., 2009). These hydrophobic interactions would then stabilize the dimer interface.

As revealed in the antagonist structure of human MD-2 and lipid IVa, all four acyl chains are accommodated inside the hydrophobic pocket of human MD-2 (Ohto et al., 2007), preventing the hydrophobic interactions necessary for dimerization in the agonist complex. However, in the crystal structure of the dimer of murine TLR4/MD-2/lipid IVa complexes, lipid IVa is bound in the same overall orientation as LPS in the complex (Ohto

et al., 2012) and a portion of an acyl chain (carbons C3→C7) protrudes from the hydrophobic pocket to make van der Waals contacts with a TLR4 residue (F438) at the dimer interface (Fig. 3.4). Due to the change in lipid IVa orientation, however, a chain on the opposite side of the lipid IVa molecule (R.2) is exposed relative to the antagonist complex (Ohto et al., 2012).

8.3. Lipid A Analogs

In an attempt to separate the beneficial immunopharmacological from the harmful pathophysiological endotoxic properties of lipid A analogs, researchers have focused on simplified structures for structure–activity studies. Based on the structure of lipid A, the general endotoxic structure is that of an amphiphilic molecule with separate polar and apolar domains of specific relative proportions and orientations. The combination of a hydrophilic backbone supporting a hydrophobic core is essential for agonistic or antagonistic activity (Seydel et al., 2003). However, isolated hydrophilic (diglucosamine) or hydrophobic (six acyl chain) domains are not active in classic toxicity or therapeutic assays, although a bisphosphorylated diglucosamine lacking acyl chains shows modest activity in the *Limulus* amebocyte lysate (LAL) clotting assay used for endotoxin detection (Takada et al., 1988). Therefore, separation of the hydrophilic and hydrophobic domains is not suitable for separation of endotoxic and beneficial activities.

Interestingly, the specific structure of the diglucosamine backbone of lipid A does not seem to be a strict determinant for biological activity. Synthetic derivatives in which one or both of the glucosamines residues were replaced with acyclic or pseudopeptide backbones have been described with similar activities to the parent lipid A molecule (Akamatsu et al., 2006; Hawkins et al., 2002; Johnson, 2008; Shimizu et al., 1995; Tamai et al., 2003). The presence of at least one, and optimally two, negatively charged phosphate bioisosteres in appropriate orientations does, however, seem to be a prerequisite for substantive activity (Przetak et al., 2003).

Alternatively, dividing the lipid A molecule into the reducing and nonreducing halves is a strategy that has been used for development of therapeutics with some success (Johnson, 2008; Perera et al., 1993). Lipid X, a biosynthetic precursor to *E. coli* lipid A, represents the reducing half of the molecule and consists of a 1-phosphoryl, diacyl (2-*N*- and 3-*O*-3-hydroxytetradecanoyl) D-glucosamine monosaccharide (Fig. 3.4a). Lipid X-like structures were found to lack both beneficial immunostimulatory and endotoxic activity in murine and human models and were often

antagonists of LPS or lipid A (Takahashi et al., 1987). However, lipid X analogs with a third acyl chain (SDZ MRL 953; Fig. 3.4a) at the 4-position of the glucosamine induced non-specific resistance to bacterial infection in mice although it was 1000-fold less potent than LPS, with minimal endotoxicity (Lam et al., 1991).

Analogs of the nonreducing half of lipid A also have weak endotoxic activity. However, some analogs, especially 4-phosphoryl D-glucosamines with three acyl chains of between 12 and 14 carbons (i.e. GLA-60; Fig. 3.4a), were found to be relatively potent adjuvants (Matsuura et al., 1995), were tumor regressive (Nakatsuka et al., 1989) and provided non-specific protection from viral or bacterial infection (Ikeda et al., 1990) in mice. 4-phosphoryl, triacyl glucosamines were also found to have relatively potent immunostimulatory activity for human cells, although the structural requirements were stricter than for mice (Matsuura et al., 1999). The low endotoxicity coupled with the potent adjuvant and immunostimulatory activity of these triacyl lipid A analogs gives them a high therapeutic index and makes them attractive targets for development.

8.4. Synthetic Monophosphoryl Lipid A

The therapeutic potential of "detoxified" lipid A analogs was initially illustrated with the isolation and characterization of 3-*O*-desacyl-4′-monophosphoryl lipid A (MPLA or MPL® from GlaxoSmithKline) from the *S. minnesota* R595 lipid A mutant (Johnson et al., 1987; Qureshi et al., 1985; Qureshi et al., 1982; Takayama et al., 1984). Detoxification was achieved by successive acid hydrolysis of the 1-*O*-phosphono group, followed by base hydrolysis of the (*R*)-3-hydroxytetradecanoyl group, yielding a mixture of monophosphorylated tetra-, penta-, and hexa-acylated congener lipid A species (Hagen et al., 1997).

Analysis of MPL® isolated by acid and base hydrolysis of LPS from Re mutants of *S. minnesota* R595 revealed the presence of a mixture of congeners that differed primarily in the character and degree of acylation. In addition to the most active hexa-acyl component, the mixture included tetra- and penta-acyl species (Hagen et al., 1997). The heterogeneity resulted from the number and length of acyl chain substitutions in the natural LPS preparation, as well as from incompleteness and lack of chemoselectivity in the alkaline hydrolysis steps. The result was an MPL isolation product composed of a mixture of congeners of 4′-phosphoryl diglucosamine with four to six acyl chains of varying length (Hagen et al., 1997; Johnson, Sowell, et al., 1999). Therefore, synthetic versions of the major congeners of in

the MPL® mixture were synthesized and tested to confirm the structure and activity of the components, as well as to study the structure–activity relationships governing beneficial activity and toxicity of the active components (Johnson, 2008).

A comparison of synthetic MPL congeners provided insight into the structural determinants of endotoxicity and adjuvanticity. In agreement with the results from that of synthetic analogs based on *E. coli* lipid A by the Osaka group (Takada & Kotani, 1989), synthetic MPL analogs with more than or less than six acyl chains were, in general, less endotoxic in vivo and induced less inflammatory cytokine in vitro than the hexa-acylated component (Johnson, 2008; Johnson, Keegan, et al., 1999). Hexa-acylated analogs were also more pyrogenic than those with fewer (4–5), or more (7) acyl chains. However, in contrast to *E. coli* hexa-acylated lipid A with an acyl chain at the 3 position, the MPL hexa-acylated analog with an SAC at the 2 position was slightly less pyrogenic than the hepta-acylated analog (Johnson, Keegan, et al., 1999), suggesting SAC distribution may also affect endotoxic response.

There was also a bimodal dependence of inflammatory cytokine induction and endotoxicity (pyrogenicity and D-galactosamine-sensitized mouse toxicity) on the length of the ester-linked SACs at the 3′, 2′ and 2 positions for synthetic MPL analogs. MPL analogs with SACs of eight carbons were the most inflammatory and endotoxic, followed by those with 6-, 10- and 12-carbon SACs, with some variations based on the species of animal treated. Inflammatory cytokine induction in murine macrophages and endotoxicity were abolished or severely reduced for analogs with less than four carbon SACs respectively, while cytokine induction was abolished below six carbon SACs in human whole blood. Interestingly, analogs with six carbon SAC were still potently pyrogenic (rabbit) and endotoxic (mouse toxicity), while inactive at inducing inflammatory cytokines in human whole blood (Johnson, Keegan, et al., 1999).

In contrast to the high inflammatory and endotoxic activity of MPL analogs with shorter SACs (6–10 carbons), compounds with longer SACs, including the analog of the "natural" hexa-acylated MPL congener with 14, 12 and 16 carbon SACs on the 3′, 2′ and 2 positions respectively, induced better antibody responses to a tetanus toxoid antigen in a murine vaccination model (Johnson, Keegan, et al., 1999). Therefore, the beneficial adjuvant and toxic effects of the analogs could be separated based upon the length of the SAC. This observation and the previous studies with *E. coli* lipid A analogs suggested that the number of acyl chains or phosphate groups had

little effect on adjuvanticity, while the length of the SAC seems to be the most important factor for determining beneficial biological activity (Johnson, 2008). However, possible mechanisms for the separation of endotoxicity and adjuvanticity based on acyl chain lengths were not postulated.

8.5. Aminoalkyl Glucosaminide 4-Phosphates

In order to further explore the determinants of the beneficial adjuvant and immunotherapeutic potential of lipid A analogs, the acylated Ω-aminoalkyl 2-amino-2-deoxy-4-phosphono-β-D-glucopyranosides, commonly referred to as the aminoalkyl glucosaminide 4-phosphates (AGPs), were developed. The project began in the 1990s at Ribi Immunochem (Johnson, Sowell, et al., 1999). Researchers at Ribi had previously developed MPLA adjuvant (MPL®) as a candidate vaccine adjuvant and immunotherapeutic (Hagen et al., 1997). Synthesis of the AGPs was a response to the need for pure lipid A congeners for studies of the mechanism of action of lipid A analogs, for determining the structure–activity relationships, and for simplifying synthesis necessary for scale-up of production.

The AGPs have the general structure shown in Fig. 3.5b. The design was based on the combination of the more conserved nonreducing glucosamine half of lipid A linked to a conformationally flexible N-acylated aminoalkyl (aglycon) residue (Johnson, Sowell, et al., 1999). There were several primary reasons for the choice of the N-acylated aminoalkyl substituent to replace the reducing glucosamine of lipid A (Johnson, 2008; Johnson, Sowell, et al., 1999). First, the conformationally flexible structure of the aminoalkyl allows the energetically favored, close packing of acyl chains that is believed to be a characteristic of potent (hexa-acyl) lipid A forms (Brandenburg et al., 1995; Seydel et al., 1993). Second, the simplified chemistry of the aglycon substituent is more amenable to alternative synthesis strategies and scale-up than the diglucosamine structure of lipid A. Third, the simpler structure of the AGPs provides several positions amenable for systematic variation of functional groups for structure–activity relationship studies.

From this basic structure, modifications were made to vary the length, number and position of up to three secondary acyl chains (R2), to vary the length of the spacer (*n*) between the nonreducing glucosamine and the N-acylated aminoalkyl, and to vary the character of the aglycon substituent (R1) (Johnson, Sowell, et al., 1999). However, as differences in the length of primary acyl chains were previously shown to have limited effects on the bioactivity of MPL analogs (Johnson, Keegan, et al., 1999), the AGPs

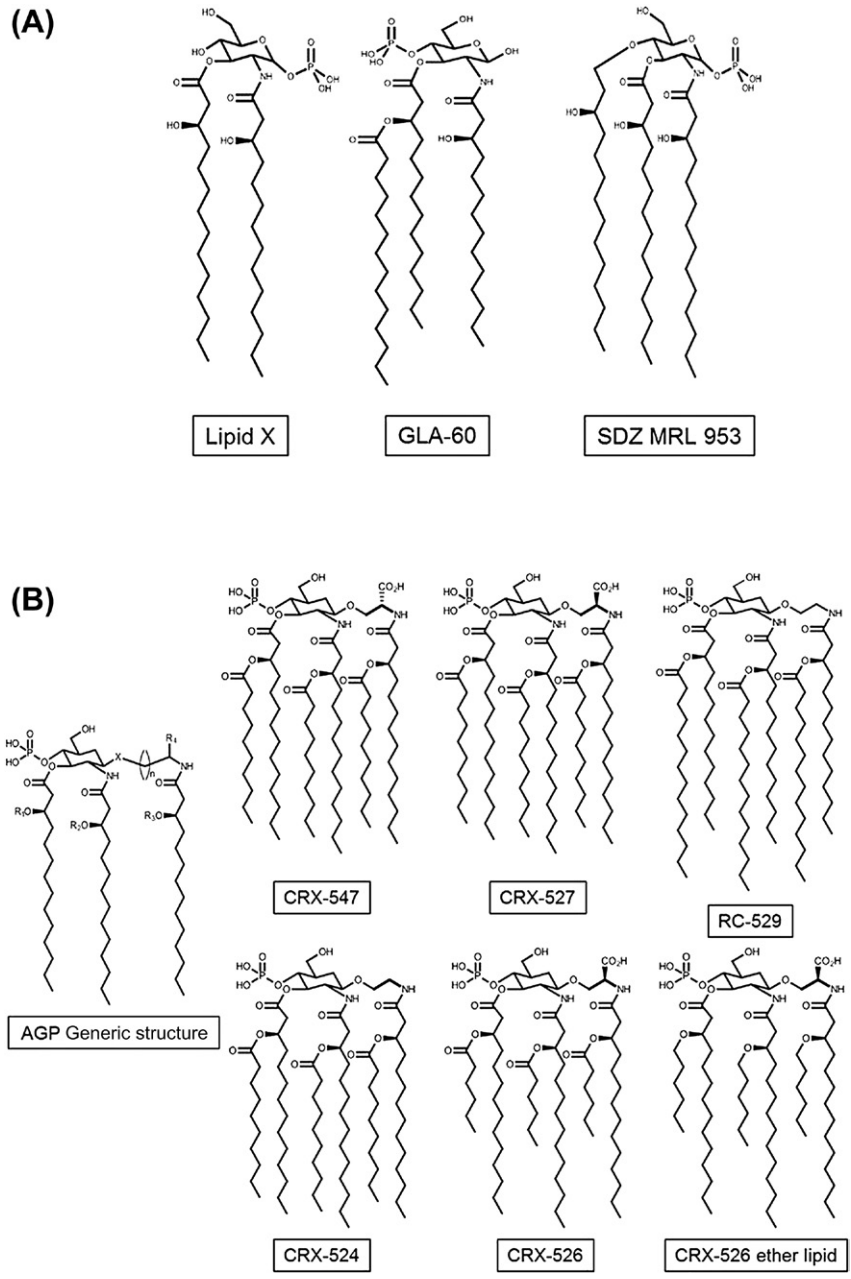

Figure 3.5 Structures of synthetic monosaccharide derivatives of lipid A. (A) Triacylated monosaccharides lipid As. (B) The aminoalkyl glucosaminide 4-phosphate (AGP) family of lipid A mimetics.

were synthesized with three (*R*)-3-*n*-alanoyloxytetradecanoyl primary acyl chains (Johnson, Sowell, et al., 1999) and three SACs of varying lengths.

As was outlined in previous sections, the length of the SACs has a substantial effect on both the bioactivity of synthetic lipid A analogs as well as the activity of natural lipid A species from pathogenic bacteria. Therefore, to determine the structural determinants for the activity differences of lipid As with differences in SAC length, Stover et al. (2004) studied the effects on biological activity by systematically changing the length of one, two or all three SACs in a series of AGP constructs. The authors found that an AGP with SAC lengths of 10 carbons (CRX-527; Fig. 3.5b) was the most potent inducer of signaling, while decreasing SAC lengths below 10 carbons (6 or 8) or increasing SAC lengths above 10 carbons (12 or 14) progressively reduced signaling through the human TLR4/MD-2 receptor. The expression of membrane-bound CD14 with the TLR4/MD-2 complex augmented but was not strictly necessary for the activity of the compounds. Surprisingly, an AGP with six carbon SACs (CRX-526, Fig. 3.5b) proved to be a potent inhibitor of signaling, the first report of a hexa-acylated TLR4/MD-2 inhibitor (Fort et al., 2005). In addition, changes in the length of the SAC at the 3′ position of the glucosamine, especially a decrease to six carbons at this position, most profoundly affected activity in human CD14/TLR4/MD-2 expressing cells. This result suggested the importance of orientation effects for binding and activation of the receptor complex.

Intravenous administration of the compounds in mice showed the same pattern of relative activities, with 10 carbon SACs being the most potent inducers of serum cytokines, followed by 12, 8 and 14 SACs. Unlike tetra-acylated compounds such as lipid IVa, CRX-526 was a potent inhibitor of signaling through the mouse complex as well as the human complex (Bazin et al., 2008).

In order to increase the chemical and metabolic stability of AGPs, several of the compounds were synthesized with ether rather than ester linkages for the secondary acyl chains (Bazin et al., 2008). An ether lipid analog of CRX-527 had virtually identical agonist activity for human and murine TLR4 receptor complex. The ether lipid analog also provided equivalent protection against *Listeria monocytogenes* or influenza challenge in murine innate immunity, nonspecific resistance (NSR) models (Bazin et al., 2008). As expected, the ether lipid analog of CRX-526 (Fig. 3.5b) was an antagonist of LPS activity on human cells. However, this analog was not an antagonist, but rather a weak agonist in murine systems. This species-specific activity is reminiscent of that seen for lipid IVa in human and mouse, and

it suggests that interactions of the secondary ester carbonyls of CRX-526 with MD-2 or TLR4 must be important for its antagonistic activity.

The crystal structure of a dimer of murine MD-2, TLR4 and lipid IVa suggests that residues on MD-2 surrounding the acyl chain-binding hydrophobic pocket and TLR4 of an interacting dimer partner complex are in close proximity and interact with the secondary carbonyl oxygen of lipid IVa (Ohto et al., 2012). Specifically, Y102 of MD-2 and R434 of the dimerization partner TLR4 appear to interact with lipid IVa carbonyl oxygen in a dimer complex of $(mTLR4/mMD\text{-}2/lipid\ IVa)_2$ (Fig. 3.4b). These interactions may help stabilize the complex in the active conformation seen for the murine $(TLR4/MD\text{-}2/lipid\ IVa)_2$ complexes, but they are lacking in the human MD-2/lipid IVa structure (Ohto et al., 2007, 2012). Thus, the presence of the secondary ester carbonyls on CRX-526 may hinder these interactions while ether-linked secondary acyl chains may not. In a similar manner, replacement of the 3-hydroxyl groups on the four acyl chains of lipid IVa with either succinyl ($COCH_2CH_2COO^-$) or acetyl ($COCH_3$) groups converted the molecule from an agonist into an LPS antagonist in murine macrophages (Tanamoto, 1995).

9. TRIF-SELECTIVE SIGNALING

9.1. Monophosphoryl Lipid A

MPL® from GlaxoSmithKline Biologicals has been successfully tested as a safe and effective adjuvant in both prophylactic and therapeutic vaccine indications (Ulrich & Myers, 1995). In fact, MPL® was the first adjuvant other than aluminum salts (alum) to be approved for use in human vaccines. MPL® was approved for use in a Hepatitis B vaccine (FENDrix–GlaxoSmithKline Biologicals) in 2005 (Europe) and for prevention of cervical cancer caused by the human papilloma virus (HPV) (Cervarix–GlaxoSmithKline Biologicals) in 2007 (Europe and Asia) and 2009 (US) (Didierlaurent et al., 2009; Kundi, 2007). In both cases, antigen was formulated with the MPL® adsorbed to alum, designated Adjuvant System-04 (ASO4), for optimal efficacy (Didierlaurent et al., 2009; Garcon et al., 2011).

Studies of the mechanisms for adjuvanticity of ASO4 suggested that MPL was chiefly responsible for the stimulation of a TLR4-dependent innate immune response that was transient and localized to the injection site, while the Alum component increased the duration of the response through induction of chemokines and a possible depot effect, retaining the antigen and TLR4 agonist together at the injection site (Didierlaurent et al.,

2009; Garcon et al., 2011). This localized response led to recruitment and activation of antigen-loaded APCs that then trafficked to draining lymph nodes to prime antigen-specific T-helper (T_h)-type T cells for subsequent differentiation of antigen-specific B cells (Didierlaurent et al., 2009). In this case, the beneficial therapeutic potential of MPL® derives from the contribution of the relatively potent stimulation of antigen-specific APCs for efficacy combined with the transient and localized nature of the response for safety.

Evaluation of a detoxified *S. minnesota* lipid A (MPLA) closely related, but not identical to MPL® from GlaxoSmithKline, suggested another potential mechanism for its favorable therapeutic potential in vaccines. In 2007, Mitchell et al. discovered that MPLA induced biased signaling through TLR4 that was characterized by repressed levels of MyD88-dependent but relatively unimpaired levels of TRIF-dependent signaling as compared to *S. minnesota* LPS (Mata-Haro et al., 2007). The authors found that mice treated with a potency-adjusted dose of MPLA expressed similar levels of TRIF-dependent cytokines and chemokines, but significantly lower levels of MyD88-dependent cytokines and chemokines as defined by expression patterns reported in MyD88 and TRIF-deficient mice or cells (Bjorkbacka et al., 2004; Kawai et al., 2001; Yamamoto et al., 2003). Intriguingly, those same mice showed equivalent levels of antigen-specific $CD4^+$ and $CD8^+$ T cell priming in an adoptive transfer model utilizing ovalbumin peptide-specific T-cell receptor transgenic mice, indicating comparable T-cell adjuvanticity. Further, MPLA stimulation of T-cell proliferation showed a much greater dependence on TRIF- than MyD88-dependent signaling in experiment with adaptor-deficient mice.

TRIF-biased signaling through TLR4 could help explain the combination of potent vaccine adjuvanticity with low toxicity in MPLA preparations compared with LPS. MyD88-dependent signaling primarily controls the gene expression of inflammatory mediators often associated with endotoxic response. On the other hand, TRIF-dependent signals primarily drive multiple mechanisms that modulate adaptive immune response. For instance, TRIF-dependent signals induce dendritic cell maturation in a type I IFN-dependent manner, leading to upregulation of MHC molecules and costimulatory molecules necessary for optimal T-cell priming (Hoebe et al., 2003; Yamamoto et al., 2003). Type I IFN also directly acts on activated T cells to promote their expansion and survival (Kolumam et al., 2005; Marrack et al., 1999). In addition, TRIF-dependent signals regulate chemokines and chemokine receptors that promote effector T-cell trafficking

(Dufour et al., 2002; McAleer et al., 2009; Weighardt et al., 2004; Yoneyama et al., 2002). Finally, TRIF acts in conjunction with MyD88 to drive the optimal production of cytokines (IL-12) that promote T-cell differentiation to Th1 phenotype (Weighardt et al., 2004).

9.2. *Salmonella minnesota* MPLA

Subsequent biological comparisons of similar synthetic versions of the hexa-acyl congener of *S. minnesota* MPLA (sMLA) and diphosphoryl lipid A (sDLA) suggested that the absence of the 1-phosphoryl group on the hexa-acylated analog was sufficient to reproduce the low inflammatory, TRIF-biased signaling character of the biologically derived MPLA preparation reported by Mata-Haro et al. (Cekic et al., 2009, 2011; Mata-Haro et al., 2007). In addition, these studies helped explain questions brought up by previous results with MPLA: if lower inflammatory signaling by MPLA could be explained by simple selection of TRIF- versus MyD88-dependent signaling downstream of TLR4, why were some TRIF-dependent genes (i.e. MCP-1) not equally induced, and why were some more MyD88-dependent inflammatory genes (i.e. TNFα) strongly induced by MPLA relative to LPS or lipid A?

As a partial answer, Cekic et al. (2009) found that TRIF-dependent signaling could still induce relatively strong signaling through the p38 mitogen-activated protein kinase (MAPK) pathway, a pathway important for regulation of inflammatory responses in general and TNFα expression specifically, while activation of the JNK MAPK was poorly induced by MPLA signaling. The JNK pathway regulates a subset of inflammatory cytokine and chemokine genes, including MCP-1, at the transcriptional level (Wolter et al., 2008). However, activation of p38 and JNK pathways was dependent upon both MyD88 and TRIF in their assays, suggesting that MPLA may stimulate partial MyD88-dependent signaling with outcomes dependent upon the particular pathway downstream of TLR4. For instance, robust induction of p38 activation may be desirable for early expression of a subset of inflammatory genes important for a potent localized innate response, but p38 activation also leads to expression of anti-inflammatory mediators such as IL-10 and phosphatases such as MAP kinase phophatase-1 (MKP-1) that, in turn, can limit the duration and scale of the inflammatory response (Guo et al., 2003; Li et al., 2009).

Cekic et al. (2011) also found that MyD88-dependent signaling by sMLA induced rapid activation of the anti-inflammatory SH2-domain containing inositol phosphatase-1 (SHIP-1) (Cekic et al., 2011). SHIP-1

is a lipid phosphatase that can counteract the effects of PI3K on phosphatidylinositol-3,4,5-triphosphate, leading to reduced production of inflammatory mediators (Haddon et al., 2009). Here, the authors found that although sMLA induced rapid recruitment of MyD88 to the TLR4 TIR domain and early activation of SHIP-1 to an extent similar to lipid A, sMLA activated some signaling pathways downstream of TLR4 relatively poorly. For instance, sMLA activated IRAK1, part of the signaling cascade downstream of TLR4 that culminates in inflammatory NF-κB and MAPK signaling, relatively weakly. Therefore, lower inflammatory signaling by sMLA could be explained by the combination of reduced signaling through select pathways downstream of MyD88 (IRAK1) with robust activation of some MyD88-dependent anti-inflammatory mediators. The mechanisms for this MyD88-dependent signaling selectivity have not yet been elucidated.

Recently, another potential mechanism for the reduced inflammatory activity of sMLA in vivo was reported. Embry et al. found that poor MyD88-dependent signaling by sMLA was responsible for a significant decrease in the secretion of active IL-1β. Although sMLA and synthetic lipid A induced comparable intracellular expression of immature pro-IL-1β, sMLA was a poor activator of the NLRP3 inflammasome responsible for activation of caspase-1. Caspase-1 is the enzyme required for cleavage of pro-IL-1β to its active and secreted form. This result could be explained by the relatively poor MyD88-dependent induction of NLRP3 expression by sMLA, leading to lower caspase-1 activation and, therefore, lower inflammatory IL-1β secretion.

9.3. CRX-547

Recently TRIF-selective signaling from a lipid A analog from the monosaccharide AGP family was described (Bowen et al., 2012). The related AGPs referred to as CRX-547 and CRX-527 contain three (R)-3-decanoyloxytetradecanoyl residues N- or O-linked to an O-glucosaminyl serine backbone. The seryl aglycon is a conformationally flexible substitute for the reducing sugar of lipid A, and the seryl carboxyl group is bioisosteric with the anomeric phosphate of lipid A. CRX-547 contains a D-seryl and CRX-527 contains an L-seryl aglycon unit. CRX-527 signaled as potently through MyD88- and TRIF-dependent pathways as LPS downstream of TLR4 in human monocytes and dendritic cells. Although CRX-547 signaled relatively robustly through the TRIF-dependent pathway, the analog signaled poorly through the MyD88-dependent pathway relative to

CRX-527 and LPS (Fig. 3.4). In a similar manner to MPLA and sMLA, reduced MyD88-dependent signaling by CRX-547 resulted in decreased secretion of potentially harmful, proinflammatory cytokines by human monocytes while production of TRIF-dependent mediators of a beneficial adaptive response remained intact.

As the stereochemical change in CRX-547 involved the carboxyl bioisostere of the phosphate that is missing in sMLA, it was perhaps not completely surprising that CRX-547 produced the TRIF-selective signaling phenotype. However, TRIF-dependent signaling by CRX-547 was qualitatively different from sMLA in human monocytes (Fig. 3.6). While sMLA was less potent than CRX-527 and LPS at inducing MyD88-dependent TNFα production, the potency was similar for induction of TRIF-dependent RANTES (not shown). With CRX-547, the maximum response of MyD88-dependent TNFα was markedly reduced relative to CRX-527 and LPS, while the potencies were similar. In fact, CRX-547 was a partial agonist for induction of MyD88-dependent TNFα from human monocytes, with substantially lower efficacy or maximum response than CRX-527 and dose-dependently inhibited CRX-527-induced TNFα production down to the maximum level induced by CRX-547 alone.

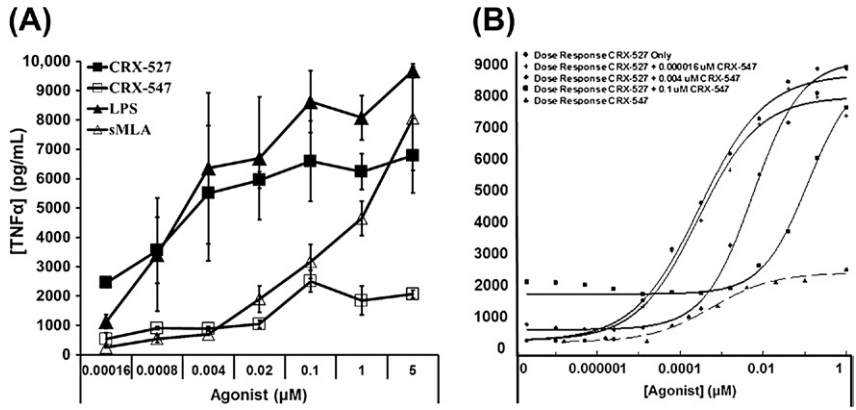

Figure 3.6 Comparison of the MyD88-dependent TNFα induction from human monocytes treated with CRX-527, CRX-547, Re595 LPS, or synthetic MLA (sMLA). (A) Human PBMC-derived monocytes were treated with agonists for 18 h and supernatants were analyzed for the presence of TNF-α. *(Data from Supplementary Figure 1 of Bowen et al., 2012 with permission.)* (B) CRX-547 inhibits CRX-527-dependent induction of MyD88-dependent TNFα. Human primary monocytes were treated with a dose response of CRX-527 following pretreatment (15 min) with one of the three concentrations (0.000016 μM, 0.004 μM, 0.1 μM) of CRX-547. *(Data from Figure 7 of Bowen et al. (2012) with permission).*

What these qualitative differences tell us about the mechanisms of selective or biased signaling by CRX-547 and sMLA is not clear. One theory of functional selectivity in receptor signaling, termed stimulus trafficking, suggests that agonists may differentially bind or induce conformations of the receptor that preferentially bind specific intracellular signaling adaptors, leading to selective signaling downstream (Kenakin, 1995). The results above suggest that sMLA retains the ability to induce a receptor conformation conducive to MyD88-dependent signaling at higher concentrations, a phenotype consistent with lower affinity binding, while CRX-547 does not efficiently induce the active conformation for MyD88-dependent signaling, even at high concentrations.

But what regulates activation of MyD88- versus TRIF-dependent signaling? Kagan et al. have proposed that activation of MyD88- and TRIF-dependent signaling is a sequential process (Kagan et al., 2008). First, the sorting adaptor TIRAP, localized to the plasma membrane through a specific interaction with phosphatidylinositol-4,5-bisphosphate PtdIns(4,5)P_2, recruits MyD88 to the activated TLR4/MD-2 complex to initiate signaling. Next, the signaling complex is endocytosed and TIRAP and MyD88 are released when the PtdIns(4,5)P_2 concentration drops in the endosomal membrane. Finally, TRIF is recruited to the TLR4 complex through an interaction with the TRAM and TRIF-dependent signaling commences (Fig. 3.1). The compartmentalization of signaling is maintained because TRAM is specifically recruited to the endosomal membrane through a bipartite (myristate group and phosphoinositide-binding domains) localization motif, and TRIF signaling depends upon an interaction with cytosol-restricted TNF receptor-associated factor-3 (TRAF3).

In the Kagan model, TRIF-dependent signaling is regulated by endocytosis, suggesting that agonists that selectively signal through TRIF may be selectively endocytosed with limited opportunities for MyD88-dependent signaling. Therefore, binding of a TRIF-selective agonist would induce a conformation of the receptor complex that is unfavorable for MyD88-dependent signaling yet favorable for endocytosis. As CD14 has recently been found to be important for endocytosis (Zanoni et al., 2011) and TRIF-dependent signaling (Jiang et al., 2005; Zanoni et al., 2011), the TRIF-selective conformation may be one that favors an interaction with CD14 on the cell surface. In subsequent experiments, we have yet to discern a clear CD14 dependence in the signaling of TRIF-selective agonists (unpublished data), although this is an area of active research.

Alternatively, endocytosis may not require an active conformation of the receptor complex, such as dimerization, for either MyD88- or TRIF-dependent signaling. What then would explain TRIF-dependent signaling in the endosome? Gangloff (2012) recently suggested a mechanism whereby a weak agonist could still signal through the TRIF-dependent pathway. In this hypothetical model, Gangloff proposes that the low-pH environment of the endosome induces protonation of histidine residues on TLR4 which, in cooperation with the increased curvature of the endosomal membrane, forces the receptor complex to adopt a conformation modeled on that of the crystal structure of TLR3 with bound double-stranded RNA (crystallized under low-pH (5.5) conditions) (Liu et al., 2008). This structure would be less dependent upon the strength of interactions of MD-2, TLR4 and bound agonist at the dimerization interface, and could bind the TRAM/TRIF adaptor complex in a manner similar to TLR3 to induced downstream signaling. In this model, the TLR4/MD-2 receptor complex and not the agonist is intrinsically TRIF-signaling selective. Histidine mutagenesis and low-pH experiments could be used to test this hypothesis.

10. CONCLUSION

Structure–activity studies using lipid A analogs have been informative for the design of synthetic lipid A mimetics for use as prophylactic and therapeutic adjuvants. It is striking how the inflammatory and endotoxic potential of the analogs can be functionally separated from the beneficial therapeutic potential based upon relatively small structural modifications. More recent studies utilizing synthetic MPLA and monosaccharide analogs have reinforced and expanded the lessons learned in the original SAR testing of *E. coli* lipid A analogs by the Shiga group in Osaka (Takada & Kotani, 1989). In parallel, expanding knowledge of the LPS modifications that pathogenic and commensal bacteria employ to avoid the potent innate response of the host have provided evolutionary evidence for the role of certain structural features in TLR4/MD-2 receptor activation. Finally, the recent successful crystallizations and structural analysis of TLR4/MD-2 agonist and antagonist structures have provided invaluable insight to help interpret the results of structure–activity screening.

The major lessons from these studies include the following. (1) Modifications of the acyl chain number, orientation or lengths away from the canonical parameters of *E. coli* lipid A (Fig. 3.3), generally result in decreased inflammatory and/or endotoxic activity. This phenomenon probably stems

from the changes in the conformation and affinity of the modified agonists in the hydrophobic binding pocket on MD-2. Binding of the canonical structure to MD-2 appears to orient *E. coli* lipid A perfectly in the MD-2 pocket to stabilize specific ionic and hydrophobic interactions at the dimerization interface. Potent, inflammatory agonists probably closely replicate this binding conformation. (2) For the same reason, changes in the number or position of negatively charged residues, either phosphates or phosphate bioisosteres, on the hydrophilic (glucosamine) backbone of lipid A analogs potentially disrupt interactions at the dimerization interface and reducing inflammatory signaling. (3) On the other hand, the stimulation of potentially beneficial adjuvant and therapeutic activities associated with adaptive immunity by lipid A analogs was substantially less conditioned on structural orthodoxy. Often less potent inflammatory or endotoxic analogs adjuvanted comparable antibody and cellular responses relative to highly inflammatory molecules.

In the case of MPLA and CRX-547, a less inflammatory phenotype correlated with the ability of the agonist to retain significant TRIF-dependent signaling, leading to lower induction of inflammatory mediators but robust adaptive priming. However, these results are also consistent with a model of TRIF-selective signaling whereby the TLR4/MD-2 receptor complex itself may have a built-in propensity toward rigorous discrimination of lipid A structures that lead to potentially dangerous inflammatory innate responses, while maintaining more plasticity for recognition of less inflammatory/endotoxic molecules to stimulating an adaptive response.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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CHAPTER FOUR

The Biology of Thymic Stromal Lymphopoietin (TSLP)

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Contents

Abstract

Originally shown to promote the growth and activation of B cells, thymic stromal lymphopoietin (TSLP) is now known to have wide-ranging impacts on both hematopoietic and nonhematopoietic cell lineages, including dendritic cells, basophils, eosinophils, mast cells, $CD4^+$, $CD8^+$ and natural killer T cells, B cells and epithelial cells. While TSLP's role in the promotion of TH2 responses has been extensively studied in the context of lung- and skin-specific allergic disorders, it is becoming increasingly clear that TSLP may impact multiple disease states within multiple organ systems, including the blockade of TH1/TH17 responses and the promotion of cancer and autoimmunity. This chapter will highlight recent advances in the understanding of

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TSLP signal transduction, as well as the role of TSLP in allergy, autoimmunity and cancer. Importantly, these insights into TSLP's multifaceted roles could potentially allow for novel therapeutic manipulations of these disorders.

1. INTRODUCTION

Thymic stromal lymphopoietin (TSLP) is a member of the interleukin (IL)-2 cytokine family and a distant paralog of IL-7 (Leonard, 2002). Murine TSLP was discovered in thymic stromal cell line supernatants that supported B-cell development (Friend et al., 1994). Like IL-7, TSLP can stimulate thymocytes and promote B-cell lymphopoiesis. Accordingly, TSLP was initially studied as a B-cell growth factor (Levin et al., 1999). A human homolog was subsequently identified, and further characterization of the cytokine revealed a four-helix bundle structure containing six conserved cysteine residues and multiple potential sites for N-linked carbohydrate addition. As discussed later, in spite of only 43% amino acid identity, human and murine TSLP share a significant degree of functional homology (Reche et al., 2001; Sims et al., 2000). During allergic inflammation, the primary producers of TSLP are epithelial cells, keratinocytes and stromal cells although recent data have demonstrated that both dendritic cells (DCs) and mast cells are capable of TSLP production (Soumelis et al., 2002; Watanabe et al., 2004; Ying et al., 2005; Kashyap et al., 2011; Moon et al., 2011).

Several groups identified a receptor capable of binding TSLP with low affinity (thymic stromal lymphopoietin receptor (TSLPR) subunit), which shares 24% identity to the common γ receptor chain (γ_c) (Pandey et al., 2000; Park et al., 2000). Upon further analyses, the functional receptor (TSLPR) was shown to include both the TSLPR subunit and the IL-7Rα chain in humans and mice (Quentmeier et al., 2001; Park et al., 2000). The functional TSLPR is expressed by a variety of hematopoietic cell populations, such as T cells, B cells, natural killer (NK) cells, monocytes, basophils, eosinophils and DCs, as well as some nonhematopoietic cell lineages such as epithelial cells (Ziegler, 2010; Reardon et al., 2011). While classified as a hematopoietin receptor based on structural homology, the TSLPR subunit contains notable differences from canonical hematopoietin receptors. The TSLPR subunit contains the conserved box1 sequence, which regulates Janus protein tyrosine kinase (JAK) binding in other cytokine receptors, but lacks the conserved box2, and contains only one tyrosine (Y) residue four amino acids from its carboxy terminus (Park et al., 2000). Additionally, it contains

a modified WSXWS motif and multiple potential *N*-linked glycosylation sites (Tonozuka et al., 2001).

2. TSLP SIGNALING

As a member of the hematopoietin receptor family, it was originally hypothesized that the TSLPR would utilize JAKs to activate signal transducers and activators of transcription (STAT) proteins downstream of the TSLPR. Indeed, TSLP stimulation of multiple cell lines leads to STAT5 phosphorylation. However, initial experiments in these cell lines showed that TSLPR signaling occurred in the absence of JAK activation, and dominant-negative forms of JAK-1 and -2 did not affect TSLP-mediated STAT5 activation (Isaksen et al., 1999; Levin et al., 1999). Several alternatives were implicated in TSLPR signaling, such as Src kinases and phosphoinositol 3 kinase (Isaksen et al., 2002). However, two recent papers have demonstrated robust and sustained activation of JAK-1 and -2 following TSLP signaling in primary human DCs and primary human and mouse $CD4^+$ T cells (Arima et al., 2010; Rochman et al., 2010). Surprisingly, unlike IL-7Rα and γ_c in IL-7 signaling, which utilize JAK-1 and -3, the TSLPR subunit bound and utilized JAK-2 in concert with IL-7Rα-associated JAK-1. These latest findings resolve a long-standing question about the mode of TSLP signaling, and show that TSLP-induced JAK activation precedes the activation of STAT proteins. In human peripheral blood-derived $CD11c^+$ DCs, TSLP stimulation activated STAT 1,3,4,5, and 6, as well as JAKs 1 and 2 (Arima et al., 2010). Similar results have been seen using mouse DCs, with the exception that no phosphorylation of Stat6 was seen (B. D. Bell, M. Kitajima and S.F. Ziegler, manuscript submitted). These data suggest that TSLP is capable of activating multiple STAT proteins. Whether TSLP utilizes similar signaling pathways in other cell lineages and how each STAT molecule contributes has yet to be elucidated.

3. TSLP-RESPONSIVE CELLS

A plethora of cell types has been shown to be capable of responding to TSLP in vivo and in vitro. These include DCs, CD4 and CD8 T cells, B cells, mast cells, basophils, eosinophils, and NKT cells. This long list of responding cell types suggests the important role of this cytokine in orchestrating the initial response to an epithelial insult. While the normal function of TSLP is likely the maintenance of Th2-type homeostasis at barrier

surfaces (Ziegler & Artis, 2010), as will be discussed below, dysregulated TSLP expression can result in the development of type-2 inflammatory responses leading to allergic disease.

3.1. Dendritic Cells

It has now become apparent that a major TSLP-responsive cellular subset in both humans and mice are myeloid-derived dendritic cells (mDCs) (Reche et al., 2001; Zhou et al., 2005). Coculture of TSLP-activated DCs with naïve syngenic $CD4^+$ T cells led to T-cell proliferation but no differentiation, suggesting a role for TSLP in $CD4^+$ T-cell homeostasis (Watanabe et al., 2004). However, when TSLP-stimulated DCs primed $CD4^+$ T cells in an antigen-specific manner (e.g. in an allogeneic culture), the resulting T cells display characteristic features of Th2 differentiated cells (production of IL-4, IL-5, IL-13, and tumor necrosis factor (TNF)α), with the exception that IL-10 production was not evident (Soumelis et al., 2002). These data suggest that TSLP-activated DCs primed for inflammatory Th2 cell differentiation. Interestingly, TSLP, in the absence of IL-12, induced OX40 ligand (CD134) (OX40L) expression on DCs, and OX40–OX40L interactions were critical for the ability of the DCs to drive Th2 cell differentiation (Ito et al., 2005). Consistent with a role in regulating Th2 cytokine responses, TSLP-activated DCs were also capable of supporting the maintenance and further polarization of $CRTH2^+$ Th2 effector memory cells (Wang et al., 2006). In contrast, autologous TSLP-activated DCs supported the expansion and functions of $CRTH2^+$ $CD4^+$ TH2 memory cells (Wang et al., 2006), but led to T-cell proliferation and elaboration of high levels of IL-2, but not IL-4, IL-5 or IL-13, when cocultured with naïve T cells (Watanabe et al., 2004).

TSLP-conditioned DCs also augmented intestinal epithelial cell (IEC)-mediated IgA2 class switching through the induction of a proliferation-inducing ligand (APRIL) (He et al., 2007). Finally, some in vitro studies have suggested a role for TSLP in the generation of tolerogenic DCs that can drive the differentiation of regulatory T cells (Tregs) (Besin et al., 2008; Iliev, Spandoni, et al., 2009; Watanabe et al., 2005), although other studies have indicated that TSLP may hinder the production and/or maintenance of forkhead box P3 $(FOXP3)^+$ Tregs in vivo in certain disease processes (Duan et al., 2010; Lei et al., 2011).

3.2. T Lymphocytes

Early work from the Leonard lab showed that TSLPR-deficient mice had normal lymphocyte numbers, but that γ_c/TSLPR double-deficient mice

had a more pronounced defect that γ_c-deficient mice along (Al Shami et al., 2004). They also showed that TSLP could drive the expansion of T and B cells when injected into γ_c-deficient mice, showing that TSLP can affect lymphoid homeostasis. Subsequent studies showed that TSLP can also act directly on $CD4^+$ T cells, and in the presence of TCR stimulation, promoted proliferation and TH2 differentiation of naïve $CD4^+$ T cells through induction of IL-4 gene transcription (Omori & Ziegler, 2007; Rochman et al., 2007). IL-4 further upregulated TSLPR on $CD4^+$ T cells, resulting in a positive-feedback loop. Although IL-4 maintained TSLPR expression on both in vitro differentiated TH2 and TH17 cells, higher TSLPR levels were present on TH2 than on TH1 and TH17 cells, which correlated with the ability of TSLP to drive the proliferation and survival of activated TH2 cells (Kitajima et al., 2011). Naïve mouse $CD8^+$ T cells also express TSLPR, though TSLPR expression is low to absent on naïve human $CD8^+$ T cells; however, following activation, TSLPR expression is upregulated on both mouse and human $CD8^+$ T cells (Akamatsu et al., 2008; Rochman & Leonard, 2008). In both $CD4^+$ and $CD8^+$ T cells, TSLP stimulation upregulated the survival protein Bcl-2 in an STAT5-dependent manner (Kitajima et al., 2011; Rochman et al., 2010; Rochman & Leonard, 2008).

3.3. B Lymphocytes

The initial studies describing TSLP demonstrated that TSLP can support B-cell lymphopoiesis (Friend et al., 1994; Levin et al., 1999). In in vitro studies, pro-B cells derived from fetal liver, but not bone marrow, responded to TSLP although pre-B cells from both origins could proliferate in response to TSLP (Vosshenrich et al., 2003). The role of TSLP in normal B-cell development or during inflammatory responses remains undefined. However, it is clear that aberrant TSLP signaling can have a significant impact on B cells, as has been demonstrated by the association of TSLPR mutations with a subtype of B cell leukemia (Chapiro et al., 2010; Roll & Reuther, 2010; Tasian & Loh, 2011). In addition, elevated systemic TSLP has been shown to lead to aberrant B-cell development and function, with both direct effects on early B cell development and indirect effects leading to autoimmune hemolytic anemia (Astrakhan et al., 2007; Iseki et al., 2012).

3.4. Innate Immune Cells

Multiple innate immune cells express the TSLPR and respond to TSLP. For example, TSLP can enhance cytokine production from mast cells, NKT

cells and eosinophils (Allakhverdi et al., 2007; Nagata et al., 2007; Wong et al., 2009). In addition, TSLP has very recently been shown to induce eosinophil extracellular traps, extrusions of mitochondrial DNA toxic granule molecules released in response to infection (Morshed et al., 2012). Finally, TSLP has also been shown to be important for the development and function of a subset of basophils (Siracusa et al., 2011). This subset is IL-3-independent, and is recruited to site of type-2 inflammation where is it speculated that they play a role in promoting Th2-type responses (Siracusa et al., 2011; Siracusa et al., 2012; Sokol et al., 2009). Thus, TSLP not only can directly promote type-2 responses through CD4 T-cell differentiation but also can influence responses through the recruitment and activation of innate immune cells capable of producing cytokines involved in type-2 inflammation.

4. TSLP-ASSOCIATED DISEASES

The variety of TSLP-responsive cell types demonstrates that TSLP can impact type-2 inflammation through a myriad of different pathways. In addition, numerous studies in both humans and mice now implicate TSLP in a growing number of different disorders beyond allergic inflammation, including infection, cancer and autoimmunity. The following sections describe the disorders associated with TSLP and what is known about the mechanisms through which TSLP may act.

4.1. Skin Disorders

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects an estimated 10–20% of infants and young children in the United States (Boguniewicz & Leung, 2011; Leung et al., 2004). Interestingly, there is suggestive evidence of linkage between single nucleotide polymorphisms (SNPs) in the TSLP gene and AD (Gao et al., 2010). In addition, while TSLP protein was undetectable in nonlesional skin in AD patients, TSLP was highly expressed in acute and chronic AD lesions (Soumelis et al., 2002). TSLP was also overexpressed in the skin of individuals with Netherton syndrome (NS), a severe skin disease characterized by AD-like lesions as well as other allergic manifestations that result from mutations in the serine peptidase inhibitor Kazal-type 5 (SPINK5) gene, which encodes the serine protease inhibitor lymphoepithelial Kazal-type-related inhibitor (LEKTI) (Briot et al., 2009).

In mice, overexpression of TSLP specifically in the skin was sufficient to induce a disease phenotype characterized by all the hallmark

features of AD (Yoo et al., 2005). In the steady state, TSLP expression in the skin appears to be negatively regulated by retinoid X receptors (RXRs) since keratinocyte-specific ablation of the RXR isotypes RXRα and RXRβ resulted in upregulation of TSLP and development of AD-like skin inflammation (Li et al., 2005). RXRs heterodimerize with many nuclear receptor partners, including the vitamin D receptor and peroxisome proliferator-activated receptors. Administration of vitamin D or its analogs upregulated TSLP and resulted in the development of dermatitis (Li et al., 2006, 2009), suggesting that vitamin D administration may result in RXR derepression and recruitment of coactivators to promote transcription. Keratinocyte-specific deletion of Notch signaling, which causes severe epidermal differentiation defects, also resulted in high systemic levels of TSLP. However, TSLP expression in this model may be due to responses to the resulting skin barrier defect rather than directly from the loss of keratinocyte-specific Notch signaling itself since wild-type and mutant keratinocytes produced similar amounts of TSLP in in vitro cultures (Demehri et al., 2008). In SPINK5 knockout (SPINK5 −/−) mice, which reproduce many of the key features of NS, the absence of LEKTI resulted in unrestrained activity of the serine protease kallikrein 5, which directly activated protease-activated receptor 2 (PAR-2) and induced nuclear factor κB (NF-κB)-mediated overexpression of TSLP without contribution of the adaptive immune system (Briot et al., 2009; Kouzaki et al., 2009). Interestingly, in SPINK5/PAR-2 double-knockout mice, TSLP expression was greatly diminished although inflammation still occurred (Briot et al., 2010). Whether the cytokine milieu differs in the absence of TSLP remains to be determined.

TSLP may influence both the initiation and progression of allergic skin inflammation, but the relative contribution to these stages and the cellular requirements may differ depending on the context. Langerhans cell (LC) migration and activation was seen in human AD lesions in situ (Soumelis et al., 2002). Furthermore, TSLP has been shown to increase the number and maturation status of migratory LCs in human skin explants cultures and to condition LCs to prime cocultured naïve $CD4^+$ T cells to adopt an inflammatory TH2 phenotype (Ebner et al., 2007). However, mouse models of AD implicate additional cell types in the initiation and promotion of AD by TSLP. A recent study by Oh et al. (2011) implicated TSLP in mediating skin fibrosis downstream of IL-13, in part through the stimulation of fibrocyte collagen production. In a model of allergic skin inflammation using epicutaneous (EC) sensitization to ovalbumin (OVA) on tape-stripped skin, TSLP acted directly on T cells during the challenge phase to

potentiate TH2 cytokine production (He et al., 2008). T cells and eosinophils were also required for TSLP-mediated dermal inflammation induced through intradermal delivery of recombinant TSLP protein (Jessup et al., 2008). In contrast, TSLP was involved in both sensitization and challenge phases of fluorescein isothiocyanate (FITC)-mediated contact hypersensitivity (CHS), since ear swelling was minimal if blockade of TSLP occurred prior to sensitization, but was only modestly reduced when TSLP blockade occurred after sensitization but prior to challenge (Boehme et al., 2009; Larson et al., 2010). While DC migration was intact in the absence of TSLP in EC sensitization, loss of TSLP signaling in the FITC CHS model was associated with reduced migration and activation of skin-derived antigen-bearing DCs. In addition, TSLP-responsive $CD4^+$ T cells were not required to induce a TH2 response in the CHS model (R.P. Larson and S.F. Ziegler, unpublished observations). In the setting of chronic high TSLP expression, skin inflammation also occurred in the absence of T cells (Yoo et al., 2005), possibly due to the ongoing stimulation of innate immune cells by TSLP.

TSLP has also been implicated in the phenomenon referred to as the *atopic march*, which describes the increased likelihood of individuals with AD of developing allergic rhinitis (AR) and asthma later in life (Bieber, 2008). Several models of induced TSLP expression in mouse keratinocytes result in subsequent allergic airway inflammation following intranasal challenge, suggesting that TSLP may be an important factor contributing to this progression from AD to AR and asthma (Demehri et al., 2009; Jiang et al., 2012; Leyva-Castillo et al., 2012; Zhang et al., 2009). While many of these methods used to induce TSLP expression result in artificially high systemic levels of TSLP that are not seen in AD patients, we have found that intradermal administration of TSLP triggers progression from AD to asthma in the absence of systemic TSLP (Han et al., 2012). In this study, TSLP was only needed during sensitization as the airway response to antigen challenge was shown to be TSLP-independent. These models, as well as approaches that allow for more specific expression or deletion of TSLP, will be helpful in identifying the cellular targets of TSLP and the mechanisms involved in the progression from AD to AR and asthma.

4.2. Respiratory Diseases

The initial report demonstrating high TSLP expression in AD and potentiation of inflammatory TH2 responses by TSLP also suggested a potential role for TSLP in allergic airway disease (Soumelis et al., 2002). This

hypothesis was supported by the demonstration that TSLP mRNA was present in human lung fibroblasts and bronchial epithelial and smooth muscle cells (Soumelis et al., 2002), and that aberrant levels of TSLP were associated with certain human respiratory disorders (Kamekura et al., 2009; Kimura et al., 2011; Semlali et al., 2010; Shikotra et al., 2011; Xu et al., 2010; Ying et al., 2005, 2008; Zhang et al., 2007). Lung epithelium and submucosal samples from asthmatics and chronic obstructive pulmonary disease (COPD) patients contained a greater number of TSLP mRNA positive cells, and bronchoalveolar lavage (BAL) samples from these patients had higher concentration of TSLP protein compared to healthy controls (Semlali et al., 2010; Shikotra et al., 2011; Ying et al., 2005, 2008). Although the level of TSLP expression can be variable in asthmatic patients, it has been shown to correlate directly with TH2 cytokine and chemokine expression and inversely with lung function (Shikotra et al., 2011; Ying et al., 2008). Increased expression of TSLP in the nasal epithelium has also been found in biopsies from AR patients and was associated with TH2 cytokine production and eosinophilic infiltration in epithelial-associated tissue (Kamekura et al., 2009; Kimura et al., 2011; Mou et al., 2009; Xu et al., 2010). Genetic studies also support a critical role for TSLP in allergic airway disease. Several SNPs at the TSLP genomic locus found across multiple ethnic backgrounds were associated with increased asthma susceptibility or protection (Bunyavanich et al., 2011; Harada et al., 2009, 2010; Hunninghake et al., 2010; Shamim et al., 2007; Torgerson et al., 2011). One such SNP present in the genomic TSLP locus creates a novel AP-1 transcription factor-binding site that could potentially lead to increased TSLP transcription (Harada et al., 2009).

A role for TSLP in human asthma has been well supported by a variety of mouse models, such as the surfactant protein C (SPC)-TSLP mouse, in which TSLP is constitutively expressed by the lung epithelium under control of the SPC promoter (Zhou et al., 2005). With increasing age, these mice developed a progressive asthma-like disease characterized by lung infiltration of eosinophils and TH2 $CD4^+$ T cells, airway remodeling and airway hyperreactivity. Disease in these mice was largely dependent on IL-4, IL-13, $CD4^+$ T cells and antigen (Headley et al., 2009; Zhou et al., 2008). $CD4^+$ T cells and antigen were also required in an acute asthma model using intranasal administration of TSLP in conjunction with antigen (Headley et al., 2009; Seshasayee et al., 2007). In addition to driving allergic inflammation in the lung following direct TSLP administration, TSLP played a crucial role in the well-established OVA/alum allergic

airway inflammation model. In this model, TSLP protein was found in the BAL and lung after intranasal OVA challenge, and disease symptoms were curtailed in the absence of TSLPR or when TSLP activity was blocked by antibody or recombinant TSLPR protein (Al Shami et al., 2005; Li et al., 2010; Shi et al., 2008; Zhang et al., 2011; Zhou et al., 2005). In an OVA-driven mouse model of AR, blocking TSLP also inhibited disease development (Miyata et al., 2008).

Most data currently point to a primary role for TSLP in the sensitization/priming stage of allergic airway disease. TSLP produced by activated human-derived lung cells stimulated human DCs to prime $CD4^+$ TH2 cell development and mast cell production of TH2-associated cytokines (Allakhverdi et al., 2007; Bleck et al., 2010; Soumelis et al., 2002). Furthermore, multiple studies have shown that TSLP-mediated DC activation was responsible for the disease phenotype observed in mouse models of asthma (Li et al., 2010; Seshasayee et al., 2007; Shi et al., 2008; Zhang et al., 2011; Zhou et al., 2005). TSLP-induced DC expression of costimulatory molecules, in particular OX40L, and DC production of TH2 chemokines, such as CCL17 and CCL21, are likely the predominant mechanisms of action (Seshasayee et al., 2007; Zhou et al., 2005). However, TSLP may also influence the challenge stage of allergic airway disease by supporting TH2 $CD4^+$ T cell cytokine production (Al Shami et al., 2005; He et al., 2008; Li et al., 2010; Miyata et al., 2008; Shi et al., 2008; Zhang et al., 2011). As mentioned above, TSLP may also influence the Treg compartment. Several reports have shown the ability of TSLP to promote the development of thymic Tregs in vitro (Hanabuchi et al., 2010; Mazzucchelli et al., 2008); however, in vivo, its role is less clear. In allergic airway disease, TSLP inhibited IL-10 mediated Treg function and the formation of inducible Tregs to exogenous antigen (Nguyen et al., 2010). Importantly, the BAL fluid from asthmatics inhibited pulmonary Treg function in a TSLP-dependent manner (Nguyen et al., 2010). In the OVA allergen model, TSLP was shown to interfere with tolerance by inhibiting the generation of allergen-specific Tregs (Lei et al., 2011). In the same model, nucleotide-binding oligomerization domain-containing protein 2 (Nod2), and to a lesser extent Nod1 stimulation blocked tolerance to OVA intranasal challenge in a TSLP- and OX40L-dependent manner (Duan et al., 2010). In this model, loss of TSLP signaling correlated with increased antigen-specific $FOXP3^+$ T cells following Nod2 stimulation.

A variety of stimuli, such as IL-4, IL-13, TNF-α, IL-1, bacterial peptidoglycan, lipoteichoic acid, double-stranded RNA (dsRNA), respiratory viruses, air pollutants and allergens have been shown to induce TSLP expression by lung-derived parenchymal cells and immune cells (Allakhverdi et al., 2007; Bleck et al., 2010; Lee & Ziegler, 2007; Kashyap et al., 2011; Kato & Schleimer, 2007; Kouzaki et al., 2009; Smelter et al., 2010; Soumelis et al., 2002; Zhang et al., 2007). In particular, stimulation of Nod1 and Nod2 in nonhematopoietic cells was potent inducers of TH2 immunity via TSLP (Magalhaes et al., 2011). These stimuli likely all drive NF-κB-dependent expression of TSLP, as was shown to occur in human lung epithelial cells (Lee & Ziegler, 2007). Furthermore, TSLP transcription was negatively regulated by 9-cis-retinoic acid via RXRs in lung cells (Lee et al., 2008). Exposure to certain infectious agents or repeated environmental irritants may prime production of TSLP, leading to TH2-mediated human disease. For example, even in the absence of known lung disease, lung samples from smokers contained increased TSLP levels as compared to nonsmokers (Ying et al., 2008). In addition, lung epithelial cells from asthmatics produced more TSLP in response to dsRNA (viral analog) stimulation in culture (Brandelius et al., 2011; Uller et al., 2010), which may explain, at least in part, why patients with asthma tend to suffer more airway dysfunction after respiratory infections compared to healthy individuals (Jackson & Johnston, 2010). This aberrant TSLP production in response to lung insults may thus influence both the susceptibility of certain individuals to develop allergic respiratory diseases such as asthma as well as the clinical complications that arise after environmental insults to the lungs of these individuals.

Collectively, these data illustrate that aberrant lung expression of TSLP is associated with human allergic airway disease and can mimic asthma-like disease in mice. According to genetic studies and in vitro analyses, lung samples from individuals with asthma or COPD produce more TSLP in response to lung insult as compared to samples from healthy individuals. Clinical trials targeting TSLP in these conditions are currently underway. According to mouse asthma models, TSLP appears to influence the sensitization stage of allergic airway responses, but a more in-depth examination of TSLP's influence on the allergic effector response is required. Where and when TSLP acts during allergic airway disease will likely explain any trial results and dictate future therapeutic design.

4.3. Intestinal Inflammation

TSLP is constitutively expressed in both the mouse and human gastrointestinal tract but can be further induced by a variety of cytokines, microbes and microbial products (He et al., 2007; Humphreys et al., 2008; Rimoldi et al., 2005; Tanaka et al., 2010; Taylor et al., 2009; Zaph et al., 2007; Zeuthen et al., 2008). Mice carrying gene deletions specifically affecting the gut mucosa provide additional clues into the regulation of TSLP expression within the gut. TSLP mRNA levels were significantly decreased in mice with intestinal epithelial-specific deletion of Dicer (Biton et al., 2011), an enzyme involved in microRNA biosynthesis, or IκB kinase-β (Zaph et al., 2007). Both these knockout mice showed increased susceptibility to infection with the mouse whipworm *Trichuris muris*. TSLP expression was also decreased in mice carrying a missense mutation in the *Muc2* mucin gene that resulted in an epithelial defect and spontaneous colitis (Eri et al., 2011). In in vitro analyses of TSLP intestinal function, human colonic or gastric epithelial-derived TSLP has been implicated in conditioning DCs to drive development of inflammatory TH2 cells (Kido et al., 2010), Tregs (Iliev et al., 2009) or T cell-independent IgA(2) class switching (He et al., 2007). While supernatants from both human and mouse IECs can condition DCs to drive Treg differentiation, the requirements for TSLP may differ in humans and mice since the presence of TSLP was required in mouse but not human IEC supernatants to drive a tolerogenic DC phenotype (Iliev, Spandoni, et al., 2009). Additional studies are just beginning to define whether and under what conditions TSLP may function in these pathways in vivo.

As is seen in atopic diseases of the skin and lung, aberrant expression of TSLP was also seen in allergic diseases of the gut. Polymorphisms in TSLP and the TSLPR were associated with the food allergy-related disorder eosinophilic esophagitis (EoE), and this association persisted when comparing EoE patients with allergic individuals without EoE (Rothenberg et al., 2010; Sherrill et al., 2010). Additionally, TSLP mRNA expression was higher in the esophagus of pediatric patients with EoE compared to controls, and was decreased in homozygotes of the protective GG minor allele for the rs3806932 SNP. Some studies suggest, however, that TSLP not only plays an important role in the promotion of TH2 responses, but is also a key player in maintaining intestinal homeostasis and modulation of TH1/TH17 inflammation. In contrast to the increased TSLP expression seen in EoE, decreased TSLP expression was seen in noninflamed colonic tissue in Crohn's disease

(CD) and ulcerative colitis (UC), the two types of inflammatory bowel disease (IBD) (Noble et al., 2010, 2008; Iliev, Spandoni, et al., 2009; Rimoldi et al., 2005). However, studies of UC have indicated that in inflamed tissue, TSLP expression is upregulated compared with noninflamed tissue from either UC patients or controls (Noble et al., 2008; Tanaka et al., 2010).

Mouse models of TH2- and TH1-type inflammation also suggest important roles for TSLP in TH2-mediated immunity, maintenance of homeostasis and modulation of TH1/TH17 responses within the gut. TSLP was required to induce diarrheal disease in a mouse model of food allergy (Blazquez et al., 2010) and protective TH2 responses to infection with *T. muris* (Zaph et al., 2007). However, TSLP was not required for oral tolerance to OVA, or for anaphylaxis and IL-4, IL-13 and IgE production following intragastric OVA/cholera toxin sensitization and challenge (Blazquez et al., 2010). Additionally, other helminths such as *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis* and *Schistosoma mansoni* still induced TH2 responses in TSLPR knockout mice, although in some cases, these responses were modified or slightly attenuated (Massacand et al., 2009; Ramalingam et al., 2009). Thus, while TSLP may promote TH2 responses in the gut, it is not absolutely required for TH2-type inflammation. In contrast to *T. muris*, both *H. polygyrus* and *N. brasiliensis* produce excretory/secretory (ES) products that acted on DCs to attenuate IL-12/23p40 production. Of note, protective TH2 responses can be induced in *T. muris* infections in the absence of TSLP following the blockade of either IFN-γ or IL-12/23p40 (Massacand et al., 2009; Taylor et al., 2009), suggesting that TSLP may play a prominent role in attenuating TH1 and TH17 responses.

Studies using mouse models of colitis have demonstrated important effects of TSLP in modulating the disease phenotype in intestinal inflammation although there have been some conflicting results. In a chemical colitis model using dextran sulfate sodium (DSS), Taylor et al. showed that mice lacking the TSLPR developed more acute weight loss and increased colonic inflammation that correlated with higher levels of IFN-γ and IL-17A within the mesenteric lymph nodes (Taylor et al., 2009). In contrast, Reardon et al. reported comparable disease onset and severity in the DSS colitis model between mice that lack TSLP signaling versus controls. However, while wild-type mice recovered after DSS withdrawal, mice lacking either TSLP or its receptor had progressive disease and weight loss (Reardon et al., 2011). Reardon et al. showed that secretory leukocyte peptidase inhibitor (SLPI) was induced in DSS colitis in wild-type mice and that this induction was lost in TSLP knockout (TSLP KO) mice. Neutrophil elastase (NE) is a

target of SLPI, and functions to degrade a number of substrates, including progranulin, a protein important in wound healing. Consistent with a role for TSLP in the inhibition of NE, TSLP KO mice displayed increased NE activity after treatment with DSS, and inhibition of NE reduced mortality in TSLP KO mice in this colitis model. While methodological differences may account for some of the discrepancies between these studies, a growing body of evidence demonstrates that differences in microbiota among various facilities can have profound effects on the development and function of the intestinal as well as systemic immune system (Gill & Finlay, 2011). Thus, further exploration of how the gut microbiota affects TSLP expression and function may be warranted.

These studies support a role for TSLP in the promotion of TH2 responses in the gastrointestinal system, but also provide important evidence that TSLP plays a key role in the maintenance of immune homeostasis within the gut. Not only does TSLP function to attenuate TH1/TH17 responses, but also acts directly on the intestinal epithelium to support wound healing in colitis. Whether TSLP also contributes to wound healing and blockade of TH1/TH17 responses at other sites remains to be determined.

4.4. Cancer

A series of recent studies have implicated TSLP in the growth and metastasis of breast and pancreatic cancer, especially those which display an increased infiltration of TH2 cells (De Monte et al., 2011; Olkhanud et al., 2011; Pedroza-Gonzalez et al., 2011). Breast and pancreatic cancer cells and cancer-associated fibroblasts have been shown to produce TSLP in response to tumor-derived inflammatory cytokines and possibly other unidentified stimuli (De Monte et al., 2011; Olkhanud et al., 2011; Pedroza-Gonzalez et al., 2011). Furthermore, treatment of DCs with supernatants from these cells induced the TH2-attracting chemokines CCL17 and CCL22, as well as upregulation of DC costimulatory molecules CD80, CD86, OX40L and TSLPR, in a TSLP-dependent manner. Additionally, these primed DCs were able to promote TH2 polarization of $CD4^+$ T cells in vitro. In support of these in vitro data, activated DCs and CCL17 and CCL22 were detected in the tumor and draining lymph nodes, but not nondraining lymph nodes of human patients (De Monte et al., 2011). Importantly, a decreased ratio of TH1/TH2 cells in human pancreatic cancer cases was associated with disease progression and was an independent prognostic marker of reduced survival (De Monte et al., 2011). While breast cancer cells with intact TSLP expression were able to induce tumor growth and metastasis in mice,

shRNA knockdown of TSLP in these cells resulted in clones with minimal growth or metastasis (Olkhanud et al., 2011). Tumor progression and metastasis of an injected breast cancer or melanoma cell line was also decreased in TSLPR-deficient mice compared to wild-type mice (Olkhanud et al., 2011).

Previous work has shown that TH2 cytokines promote disease progression through increased survival of cancer cells, M2 macrophage differentiation, and fibrosis (collagen degradation and synthesis) (Aspord et al., 2007; Joyce & Pollard, 2009; Mantovani et al., 2008; Wynn, 2004). TSLP may be linked to these phenomena in some human cancers, possibly based on its ability to drive TH2 differentiation and M2 macrophage differentiation ((Ziegler, 2010) and Han, H. and Ziegler, S.F., manuscript submitted). Alternatively, TSLP may promote tumor progression by controlling Treg migration. CCL22 production in human breast cancer is involved in the influx of tumor Tregs that may then alter the immunoregulatory environment (Gobert et al., 2009; Ménétrier-Caux et al., 2009). Further investigation is needed to identify the important sources and targets of TSLP within the tumor environment.

In addition to the association of TSLP with certain solid tumors, the TSLPR has been shown to be overexpressed in 5–10% of childhood B-cell progenitor acute lymphoblastic leukemia (ALL) cases and approximately 60% of ALL cases in children with Down's Syndrome (Ensor et al., 2011; Mullighan et al., 2009; Roll & Reuther, 2010; Russell et al., 2009; Tasian & Loh, 2011). Approximately 15% of adult and high-risk pediatric B cell ALL (B-ALL) that lack characteristic rearrangements demonstrated TSLPR overexpression (Yoda et al., 2010). In addition, some cases of activating TSLPR mutations were found (Chapiro et al., 2010). In almost all cases, TSLPR overexpression was associated with intrachromosomal deletion or rearrangement of the TSLPR/cytokine receptor-like factor 2 (CRLF2) locus with the immunoglobulin heavy chain (IGH) locus, placing TSLPR/CRLF2 under alternate transcriptional control downstream of the P2YR8 promoter (Mullighan et al., 2009; Russell et al., 2009; Yoda et al., 2010). These rearrangements were highly correlated with the presence of JAK2 mutations and were associated with a poor prognosis (Cario et al., 2010; Ensor et al., 2011; Harvey et al., 2010; Mullighan et al., 2009; Roll & Reuther, 2010; Russell et al., 2009; Yoda et al., 2010). In murine Ba/F3 cells, expression of TSLPR and JAK2 mutant alleles promoted growth factor-independent growth (Mullighan et al., 2009; Yoda et al., 2010). Mice with systemic overexpression of TSLP may provide a model for understanding the signaling

mechanisms involved. In particular, loss of keratinocyte-specific Notch signaling resulted in high systemic levels of TSLP which correlated with a rapid expansion of pre-B cells in the early postnatal period that contributed to early mortality in these animals (Demehri et al., 2008). Interestingly, overexpression of TSLP early in the postnatal period was sufficient to drive a B cell lymphoproliferative disorder, but administration or induction of TSLP after postnatal day 14 was not although other studies have shown expansion of B-cell compartments following TSLP expression in adult mice (Astrakhan et al., 2007).

The association of TSLP and TSLP signaling pathways with hematologic malignancies as well as solid tumors implicates TSLP/TSLPR in numerous regulatory pathways that support cell growth and survival in cancer. In B-ALL, activation of signaling pathways downstream of TSLP directly promotes the growth and survival of malignant cells, whereas in breast and pancreatic cancer, TSLP likely contributes to multiple components of the tumor environment that affect growth and metastasis as well as immune evasion. Several reports suggest that TSLP/TSLPR may be useful as a prognostic marker and may present a novel target for therapeutic intervention in cancer.

4.5. Other Autoimmune Diseases and Issues of Tolerance

Mouse models with constitutive or inducible overexpression of TSLP have demonstrated that TSLP can be associated with autoimmune phenomena. TSLP overexpression in these mice was associated with the development of cryoglobulinemic glomerulonephritis due to increased production and kidney deposition of systemic polyclonal IgM and IgG via a monocyte/macrophage dependent mechanism (Astrakhan et al., 2007; Taneda et al., 2001). In addition, these mice developed red blood cell-specific autoantibodies and autoimmune hemolytic anemia in a $CD4^+$ T cell and IL-4-dependent manner (Iseki et al., 2012). Whether TSLP is involved in human mixed cryoglobulinemia or autoimmune hemolytic anemia is unknown.

As discussed earlier, TSLP expression was decreased in IBD, a disorder that is thought to arise due to inappropriate immune activation against normally harmless microflora. Additionally, loss of TSLP signaling in a mouse model of autoimmune gastritis resulted in more severe disease (Nishiura et al., 2012). Although the impact of TSLP on colitis in mice appears more complex (Reardon et al., 2011; Taylor et al., 2009), this supports a model in which loss of TLSP, which can block TH1/TH17 responses, leads to increased inflammation. However, data from humans and mouse models suggest that TSLP may actively promote inflammation

in TH1/TH17-associated autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS). In a proteoglycan-induced arthritis mouse model of RA, TSLPR-deficient mice had reduced immunopathology associated with decreased levels of production of IL-17, IL-1β, and IL-6, but increased IFNγ and IL-10 (Hartgring et al., 2011). Furthermore, blocking TSLP in a collagen-induced arthritis model ameliorated disease, while administering recombinant TSLP protein exacerbated disease (Hartgring et al., 2011; Koyama et al., 2007). Increased synovial concentrations of TSLP, as well as TNFα, have also been seen in synovial fluid from RA patients compared to samples from patients with osteoarthritis. In in vitro studies, TSLP-primed human myeloid DCs induced proliferation of self-reactive $CD4^+$ T cells capable of TH1 or TH2 differentiation, and TSLP priming of DCs, in conjunction with toll-like receptor (TLR)3 ligand, supported TH17 differentiation (Koyama et al., 2007; Tanaka et al., 2009; Watanabe et al., 2004). Thus, although the role of TSLP in RA is largely undefined, these data provide intriguing evidence of its possible involvement.

SNPs in the IL-7Rα gene locus have been associated with multiple sclerosis (MS) and altered Treg numbers or function (Gregory et al., 2007; Lundmark et al., 2007). While TSLPR pairs with IL-7Rα and TSLP can affect Treg development, neither disease has yet been directly linked to TSLP. However, administration of TSLP or TSLP-treated bone marrow-derived DCs into nonobese diabetic mice prevented the development of diabetes in these mice (Besin et al., 2008), suggesting a possible role for TSLP in disease therapy. Although the mechanisms involved in protection from diabetes have not been determined, protection was associated with an increased number of Tregs.

One final link that has been made between TSLP and immune tolerance is in maternal–fetal tolerance during pregnancy (Li & Guo, 2009). TSLP was produced and secreted by first semester trophoblasts, and tissue from normal pregnancies demonstrated a TH2 bias and higher levels of TSLP expression than samples from miscarriages (Guo et al., 2010; Pu et al., 2012; Wu, Guo, Jin, Liang, & Li, 2010). Thus, while TSLP expression and a TH2 bias may lead to disease progression in cancer, TSLP may contribute to tolerance at the maternal–fetal interface.

5. CONCLUSION

Much progress has been made in the understanding of TSLP biology and its role during TH2-type inflammation. Multiple cell lineages express the functional TSLPR that helps drive the immune response. More recent data have illustrated that TSLP is also involved in numerous disorders

beyond just allergy, and may play a role in maintaining homeostasis in diseases such as IBD or in disease progression in cancer and autoimmunity. In order to utilize the knowledge gained about TSLP's biological effects, a better understanding of cell-specific signaling pathways must be delineated. Of utmost importance is deciphering whether TSLP invokes similar signaling pathways within different cells. Knowledge of the key targets and sources of TSLP in different disease states will also be important in furthering our comprehension of the pathophysiology of TSLP-associated disorders. Tools that can address these questions, such as approaches that use conditional deletion of the TSLPR and cytokine, will be important in the continued investigation of the role of TSLP during both atopic and nonatopic conditions.

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ABBREVIATIONS

TSLP Thymic stromal lymphopoietin
TSLPR Thymic stromal lymphopoietin receptor
IL-7Rα Interleukin-7 receptor alpha
JAK Janus kinase
STAT Signal transducers and activators of transcription
γ_c Common γ receptor chain
AD Atopic dermatitis
NK Natural killer
SNP Single nucleotide polymorphism
NS Netherton syndrome
SPINK5 Serine peptidase inhibitor Kazal-type 5
LEKTI Lymphoepithelial Kazal-type-related inhibitor
RXR Retinoid X receptor
PAR-2 Protease-activated receptor 2
LC Langerhans cell
EC Epicutaneous
FITC Fluorescein isothiocyanate
CHS Contact hypersensitivity
AR Allergic rhinitis
COPD Chronic obstructive pulmonary disease
BAL Bronchoalveolar lavage

SPC Surfactant protein C
FOXP3 Forkhead box P3
EoE Eosinophilic esophagitis
CD Crohn's disease
UC Ulcerative colitis
IBD Inflammatory bowel disease
DSS Dextran sulfate sodium
SLPI Secretory leukocyte peptidase inhibitor
NE Neutrophil elastase
ALL Acute lymphoblastic leukemia
B-ALL B cell ALL
CRLF2 Cytokine receptor-like factor 2
TNF Tumor necrosis factor
OX40L OX40 ligand (CD134)
TLR Toll-like receptor
APRIL A proliferation-inducing ligand
IEC Intestinal epithelial cell

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CHAPTER FIVE

Eicosanoids in Metabolic Syndrome

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Abstract

Chronic persistent inflammation plays a significant role in disease pathology of cancer, cardiovascular disease, and metabolic syndrome (MetS). MetS is a constellation of diseases that include obesity, diabetes, hypertension, dyslipidemia, hypertriglyceridemia, and hypercholesterolemia. Nonalcoholic fatty liver disease (NAFLD) is associated with many of the MetS diseases. These metabolic derangements trigger a persistent inflammatory cascade, which includes production of lipid autacoids (eicosanoids) that recruit immune cells to the site of injury and subsequent expression of cytokines and chemokines that amplify the inflammatory response. In acute inflammation, the transcellular synthesis of antiinflammatory eicosanoids resolve inflammation, while persistent activation of the autacoid-cytokine-chemokine cascade in metabolic disease leads to chronic inflammation and accompanying tissue pathology. Many drugs targeting the eicosanoid pathways have been shown to be effective in the treatment of MetS, suggesting a common linkage between inflammation, MetS and drug metabolism. The cross-talk between inflammation and MetS seems apparent because of the growing evidence linking immune cell activation and metabolic disorders such as insulin resistance, dyslipidemia, and hypertriglyceridemia. Thus modulation of lipid metabolism through either dietary adjustment or selective drugs may become a new paradigm in the treatment of metabolic disorders. This review focuses on the mechanisms linking eicosanoid metabolism to persistent inflammation and altered lipid and carbohydrate metabolism in MetS.

1. INTRODUCTION

Eicosanoids represent a diverse group of bioactive lipids synthesized from polyunsaturated fatty acids (PUFA) to either proinflammatory omega-6 arachidonic acid (AA) or anti-inflammatory omega-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Fig. 5.1). These eicosanoids are synthesized from two essential fatty acids (FAs), ω-6 linoleic acid (C18:2n6) and ω-3 linolenic acid (LA) (C18:3n3), by a series of desaturase and elongase enzymes. Both eicosanoids and FAs are partitioned to different organelles by fatty acid transport proteins (FATPs), which transport fatty acid-coenzyme A (CoA) (FA-CoA) or fatty acid binding protein (FABP) that transports free fatty acid (FFA). The FFA is esterified by a group of organelles and FA

chain-length-selective acyl-CoA synthetase (ACS) and then incoproation of saturated FA into the sn-1 position or unsaturated FA into sn-2 position of triacylglycerol (TAG) or phospholipids (PLs) by acyltransferase. These bioactive FAs are stored in membranes as PLs [e.g. phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol] or in the endoplasmic reticulum (ER) or lipid droplets (LDs) as TAG. PLs are polar ionic lipids composed of 1,2-diacylglycerol and sn-3 phosphodiester bridge that links the glycerol backbone to usually a nitrogenous base, choline, serine, ethanolamine inositol or glycerol, while TAG has FAs located at all positions of the glycerol backbone. The release of both saturated and unsaturated FAs from PL or TAG are performed by a group of phospholipases. Phospholipidase A1 (PLA_1) releases saturated palmitic acid (C16:0) from the sn-1 producing 2-acyl lysophospholipid. Phospholipase A_2 releases unsaturated fatty acid (uSFA) either oleic acid (C18:1n9) or AA from the sn-2 position forming 1-acylphospholipid. Phospholipase C (PLC) hydrolyzes inositol PLs to yield inositol phosphates and diacylglycerol (DAG) as secondary messengers, while phospholipase D produces phosphatidic acid (PA), which is acted upon by PA phosphohydrolase to produce DAG. Lipid peroxidation of membrane PL uSFAs at the sn-2 is removed by PLA_2 producing sn-2-lysoPL that is reacylated by either arachidonyl-CoA transacylase or by an exchange reaction catalyzed by lysolecithin:lecithin acyltransferase, which is a major mechanism in membrane remodeling.

The release of AA, EPA or DHA by PLA_2 is the initial rate-limiting step in the synthesis of bioactive eicosanoids, prostaglandins (PGs), leukotrienes (LTs), and cytochrome P450 metabolites. Although the cyclooxygenase and lipoxygenase (LOX) pathways that produce prostanoids and LTs, respectively, have profound roles in inflammation and regulation of metabolism, the cytochrome P450 epoxygenase and FA omega hydroxylase P450 produce unique eicosanoids that also play a significant role in inflammation and recently, in the regulation of metabolism. The interrelationship between eicosanoid metabolic enzymes and drug-metabolizing enzymes is evident from: 1) many of the same transports for drug metabolites are also used in the trans-cellular synthesis of bioactive eicosanoids, 2) conjugation of drugs with glutathione for transport and synthesis of LTs conjugate to glutathione are performed by glutathione-S-transferase as well as glutathione being necessary for the synthesis of eicosanoids and epoxide hydrolase function in both pathways, and 3) both thromboxane synthase (TXAS) (CYP5), prostacyclin synthase (CYP8), epoxygenase CYP2 and FA omega hydrolase (CYP4) cytochrome P450 enzymes participate in drug metabolism and eicosanoid pathways.

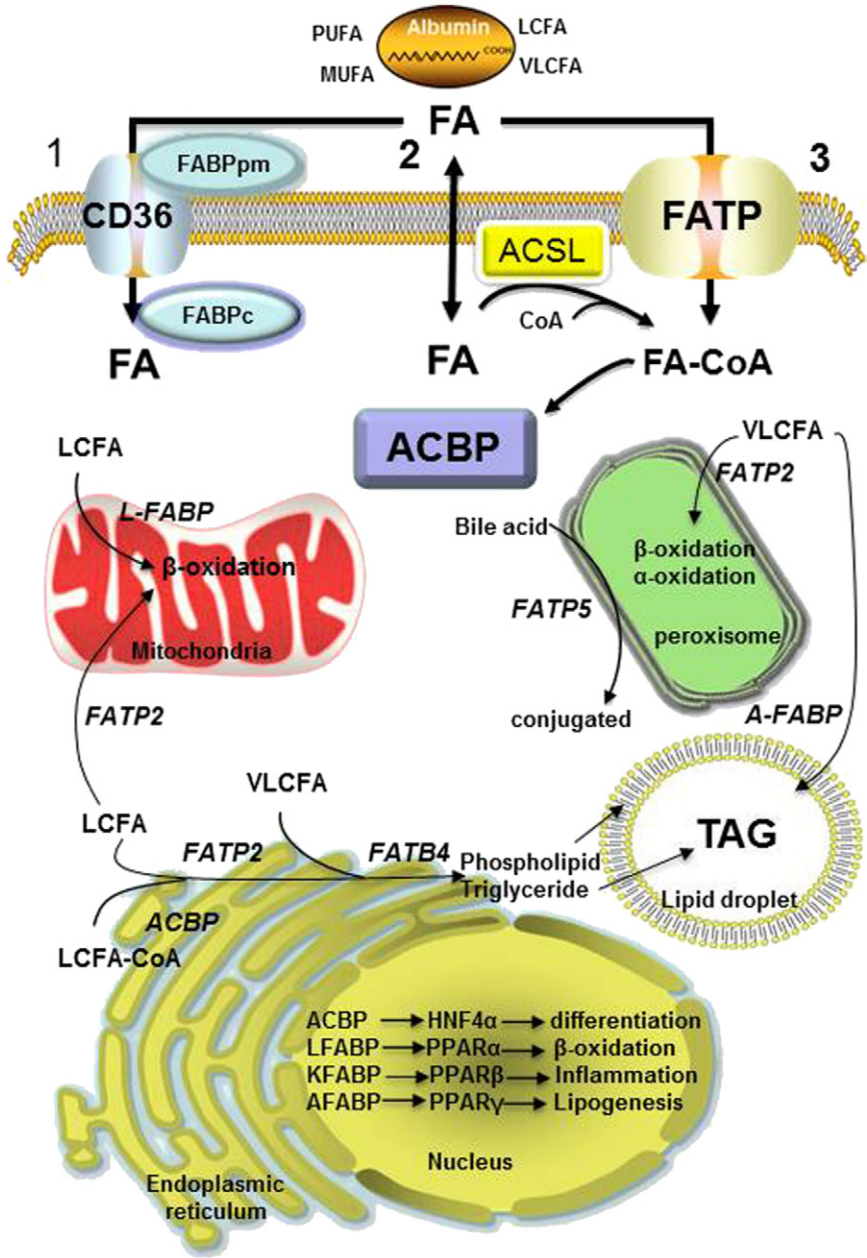


Figure 5.1 ***Function and subcellular location of fatty acid transport proteins, FABP, FATP/ACSVL, and ACBP in fatty acid transport and channeling.*** The extracelluar concentration of fatty acids (FA) varies from 0.3 to up to 2 mM and they are largely bound to albumin (300–600 uM) at a ratio of 5–10 FA molecules per molecule of albumin. Cellular

The functional role of eicosanoids in the inflammatory etiology of diseases of metabolic syndrome (MetS) has been extensively studied in relation to immune cell recruitment and cytokine, chemokine production and their activation of inflammatory pathways in cancer, diabetes, and cardiovascular disease (CVD). However, the role of eicosanoids in the regulation of metabolic pathways of lipid and carbohydrate metabolism in obesity, hyperlipidemia, hypertriglyceridemia, hypertension, and insulin resistance has only recently been studied with the use of eicosanoid metabolic enzyme transgenic and global knockout mouse models. These studies in PLA_2, 5-lipooxygenase, and 12/15-LOX pathways and knockout mice of fatty acid desaturase (FADS) and elongase (Elovl) in the formation of AA from α-linoleic acid (ALA) have strongly supported eicosanoids as key regulatory molecules in MetS and the progression of hepatic steatosis to steatohepatitis in nonalcoholic fatty liver disease (NAFLD). Furthermore, it is uncertain whether these knockout mice will show alterations in drug-metabolizing enzyme function and regulation. This will be of particular interest with respect to drugs that target inflammation through inhibition of eicosanoid metabolism. These same drugs also target key enzymes in intermediary metabolism and are metabolized by drug-metabolizing cytochrome P450s. The future challenges will include construction of floxed tissue-specific knockout animals to study the role of eicosanoid metabolism in regulation of adipose tissue lipogenesis and lipolysis, in the regulation of pancreas hyperinsulinemia and β-cell destruction, and in progression of steatosis to steatohepatitis and fibrosis in NAFLD.

uptake of long-chain fatty acid (LCFA), very-long-chain fatty acid (VLCFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) occurs through three putative mechanisms: (1) FABPpm (FABPAST) localizes FA to the plasma membrane and CD36 fatty acid translocator facilitates transport across the phospholipid bilayer and is bound by FABPc (L-FABP), (2) FA can cross the membrane by simple passive diffusion or use a flip-flop mechanism resulting in donation of proton to the cytosol. The FA can be bound to 10 different FABPs or converted to FA-CoA by acyl-CoA synthetase (ACSL) to form an acyl-CoA ester, and finally (3) VLCFA are preferentially transported by one of five FATPs that because of their synthetase activity converts VLCFAs to VLC-acyl-CoA esters. In the cytosol, FA and FA-CoA esters are channeled to different organelles and metabolic pathways by FABP and ACBP. Different FABP, FATP, and ACBP show differential selectivity for FAs of different chain length and degree of unsaturation as well as vectorial channeling to different organelles for oxidation or synthesis of complex lipids. The plasma membrane FABPpm is identical to the mitochondrial membrane aspartate aminotransferase (AST) and ths is often identified as FABPAST. Thus FABPpm has different functions depending on cellular location. Similarly, the cytosolic FABPc is also known as L-FABP and has two FA-binding sites, while other FABPs have a single FA binding site. (For color version of this figure, the reader is referred to the online version of this book).

Finally, we can learn much from patients with sepsis, glucocorticoid disorders and MetS in understanding how eicosanoids link inflammation, drug metabolism and diseases of MetS. The cardinal signs of acute inflammation of dolar (heat), calor (pain), rubor (redness), and tumor (edema) are initiated by a cascade of eicosanoid lipid autacoids, cytokines, and chemokines. Normally, the resolution of inflammation begins with the transcellular synthesis of antiinflammatory lipoxins (LX) and resolvins between different cell types. However, in chronic inflammation, the persistent cellular damage by foreign agents amplifies the inflammatory cascade, which initiates a poorly calibrated immune response that progresses from a local to a systemic response involving multiple organs, leading to immune system repression of drug metabolism and deregulation of basic metabolism. This anomaly is observed in sepsis and septicemia with multiple organ failure, which is the leading cause of surgical deaths, with a death rate equal to that of myocardial infarction. Sepsis is characterized by multiple and systemic changes in several organs that lead to insulin resistance, dyslipidemia, cholestasis, hyperbilirubinemia and vasodilation, vascular leakage, hypovolemia and coagulopathy. These symptoms are also observed in patients with Cushing syndrome and individuals with MetS. Recent studies have revealed alterations in eicosanoid metabolism in septic patients and a downregulation of the major drug-metabolizing cytochrome P450s such as CYP1, CYP2, and CYP3 families that metabolize more than 90% of known drugs, thereby making sepsis a challenge to manage from a therapeutic perspective. It is of interest that CYPs involved in the metabolism of endogenous lipids and eicosanoids have not been characterized with respect to sepsis and metabolic alterations.

The purpose of this review is not to recapitulate the several excellent reviews on eicosanoid metabolism and inflammation but to attempt to link the eicosanoids as pivotal lipid mediators in the control of inflammation and intermediary and drug metabolism in diseases such as MetS, dyslipidemia, hypertriglyceridemia, hypertension, insulin resistance and obesity. We hope that this review will provide insight into the function of eicosanoid metabolites in the regulatory control of lipid and carbohydrate metabolism in adipose tissue, pancreas, liver, and cardiovascular system under MetS.

2. LIPID METABOLISM IN CONTROL OF EICOSANOID SYNTHESIS

2.1. Fatty Acid Transporters

The uptake of essential FFAs, ALA, LA occurs through several transport mechanisms that include caveolins of lipid rafts, FATPs, FABPs, acyl-CoA

binding proteins (ACBPs), solute ligand carriers (SLCs), and fatty acid translocases (FATs/CD36) (Table 5.1). The FATPs consist of several integral plasma membrane proteins that show both chain-length and saturation-dependent transport of FFAs (Table 5.1). FATPs have ACS activity and therefore trap FAs inside the cell. FAT/CD36 is expressed in numerous tissues and facilitates FA uptake from serum albumin and insertion into membrane with assistance of FABP. Both FABP and ACBP bind an array of FAs and eicosanoids and function in the intracellular transport of both FAs and FA-CoAs to different organelles including the nucleus (Makowski & Hotamisligil, 2004). The SLC proteins are involved in the uptake of particular PG and other eicosanoids by SLC transporters that have a significant role in the transcellular synthesis of antiinflammatory eicosanoids during the resolution of inflammation (Fig. 5.1).

2.1.1. FATP

The FATP protein family is composed of six members with different preferences for saturated, branched chain and unsaturated FAs. FATP1 (SLC27A1-ACSVL4) is expressed in several tissues including adipocytes and is involved in insulin-induced membrane translocation. Overexpression of this protein increases diacylglyerol transferase activity, indicating that this transporter channels FAs to TAG synthesis (Watkins, 2008). FATP2 (SLC27A2-ACSVL1) is localized in the peroxisome and ER. This protein is believed to have a significant role in the synthesis of TAG and PL by trafficking PUFAs into both phosphatidylcholine (PC) and PI.

FATP3 (SLC27A3-ACSVL3) transports FAs to the microsomes where the FAs associate with cytosolic LDs involved in neutral lipid storage, an early event in hepatic steatosis (Poppelreuther et al., 2012). FATP3 accounts for 30% of activated intracellular FAs, and knockdown of this protein by specific siRNA significantly reduced FA uptake and synthesis of neutral lipids and LD formation. FATP4 (SLC27A4-ACSVL5) knockdown reduces the levels of PLS, cholesterol esters and ceramide in the skin leading to keratinocyte hyperproliferation and hyperkeratosis due to reduced incorporation of very-long-chain ω-hydroxylated FA into ceramide. FATP4 is associated with several organelles and is upregulated in obesity and insulin resistance in humans (Gertow et al., 2004). FATP5 (SLC27A5-ACSVL6) is the major liver transporter of bile acids, while FATP6 (SLC27A6-ACSVL2) is expressed in the placenta and heart. Three FATPs, FATP3, 4, and 6, have a preference in the transport of AA and their association with the ER indicates that they channel FAs for PL synthesis.

Table 5.1 Nomenclature and properties of fatty acid transport proteins

Gene id	Nomenclature	Tissue	Regulation	Substrate, ligand or binding protein	Subcellular location	Function
SLC27A1	FATP1-ACSVL4	Heart, adipose, muscle, brain	PPARγ	C16:0, C18:1, C24:0	M, PM, ER	β-oxidation TAG synthesis
SLC27A2	FATP2-ACSVL1	Liver, kidney	PPARα, PPARγ	C16:0, C24:0 Phytanic acid, pristanic acid	ER, P	TAG synthesis β-oxidation
SLC27A3	FATP3-ACSVL3	Kidney, ovary, lung, brain, adrenal, testis		C16:0, C18:1, C24:0	Cytosolic vesicles	Unknown
SLC27A4	FATP4-ACSVL5	Liver, kidney, heart, adipose, skin, muscle, small intestine	PPARγ, SREBP1	C16:0, C24:0	ER, P	TAG synthesis β-oxidation
SCL27A5	FATP5-ACSVL6	Liver		Cholate, THCA Chenodeoxycholate Lithocholate, C24:0 Deoxycholate	ER, P	Bile acid conjugation Bile acid synthesis
SCL27A6	FATP6-ACSVL2	Heart, placenta		C18:1, C20:4, C24:0	PM	Unknown
FABP1	L-FABP	Liver, intestine	PPARα, HNF4α	Acyl-CoA, PPARα,γ	Cytosol, N	
FABP2	I-FABP	Intestine		Acyl-CoA	Cytosol	TAG synthesis
FABP3	H-FABP	Heart, kidney muscle, thymus	c/EBPα, SREBP1 AP-1	Acyl-CoA, PPARα	Cytosol	β-oxidation
FABP4	A-FABP	Heart, adipose, epidermis, nerve	cJun, PPARγ	Acyl-CoA, PPARγ	Cytosol	Chylomicron
FABP5	E-FABP	Eye, adipose	PPARδ	Acyl-CoA, PPARβ	Cytosol	Lipogenesis
FABP6	Il-FABP	Ileum		Acyl-CoA, FXRα	Cytosol	

FABP7	B-FABP	Liver, brain	POU	Acyl-CoA		
FABP8	N-FABP	Myelin		Acyl-CoA	Cytosol	Vesicle assembly
FABP9	T-FABP	Testis	—	Acyl-CoA	Cytosol	
FABP12	R-FABP	Retina, testis	—	Acyl-CoA	Cytosol	
ACBP	L-ACBP	Liver, multiple tissues	PPARα, c/EBPα SREBP1c, Sp1 PPARγ	C14:0-C22:0 CoA esters, HNF4α	Cytosol	Glycerolipid, cholesterol synthesis
ACBP	T-ACBP	Testis, adrenal	—	C14-C22 CoA esters	Cytosol, ER	
ACBP	B-ACBP	Brain	—	C14-C22 CoA	Cytosol	
ABCP	aACBP	Adipocytes	PPARγ	—	—	FA transport
FAT/ CD36		Multiple tissues	—	Numerous	PM, M	FA transport
ACSL1	ACS1	Adipocytes	PPARα, PPARγ	C16-C24, C18:1-3,AA	PM, N,	B-oxidation
ACSL3	ACS3	Adipocytes	LXRα, PPARβ	C14-C22.C18:1-3	M, LD	TAG synthesis
ACSL4	ACS4	Liver	PPARα, SREBP1	AA, C14-C18	LD, lipid raft	
ACSL5	ACS5	Liver	SREBP1	C16-C24,C18:1-3,AA	ER, P, LD	TAG synthesis
ACSL6	ACS2	Red blood cell	—	C14-C24,C18:1-3	M, PM, ER, LD Lipid raft	PL synthesis
FABPpm	AST	Multiple tissue	—	Numerous	PM, M	FA transport

PM, plasma membrane; ER, endoplasmic reticulum; M, mitochondria; LD, lipid droplets; N, nucleus; P, peroxisome; AA arachidonic acid.
Characteristics of fatty acid transport protein (FATP-ACSVL), fatty acid binding protein (FABP) and acyl-CoA binding protein (ACBP). This table summarizes tissue-specific expression, regulation by transcription factors, substrate, ligand binding and interaction with nuclear receptors and putative function in the metabolism of fatty binding proteins.

The uptake of FAs plays a central role in metabolic homeostasis that is controlled between organs to balance storage with metabolic needs during the fed and fast states. FATPs/SLC27A1-6 not only mediate the organ-specific uptake of FAs but also functions in the intracellular partitioning of selective FAs to different subcellular location. The interplay of organ-specific uptake of FAs is apparent from knockdown of FATP5 and FATP2 in the liver and white adipose tissue (WAT). Liver-specific knockdown of FATP5 and FATP2 not only decreases long-chain fatty acid (LCFA) uptake but also prevents diet-induced hepatic steatosis through lowering hepatic TAG and LD formation, resulting in improved liver morphology, insulin sensitivity, and glucose homeostasis (Kazantzis & Stahl, 2012). Similarly, mice with adipocyte-specific knockdown of FATP1 are resistant to diet-induced diabetes and insulin resistance because of redistribution of LCFA to liver for β-oxidation. The importance of FATP in human MetS is apparent from polymorphisma in the FATP5 promoter that are strongly associated with liver disease susceptibility (Bu & Mashek, 2010) and an intron polymorphism of FATP1 that is associated with increased plasma triglycerides, chylomicrons, and low-density lipoprotein (LDL) particle size. Whether these polymorphisms influence expression of hepatic FATP5 or adipose FATP1 will have to be determined.

2.1.2. FABP

In contrast to FATP acyl-CoA synthetase activity, the 10 FABP proteins and three ACBP proteins function to distribute acyl-CoA FA between different organelles. There are two groups of FABPs, the plasma membrane FABPS that associate with CD36 and the intracellular FABPs. FABPs are named according to the tissue from which they have been isolated and function as chaperones of FA-CoA to specific organelles. FABPs are involved in the conversion of FAs to eicosanoids, saturation and transport of LTA_4 in transcellular metabolism during resolution of inflammation, as well the transport of the PUFAs, DHAs and lysoPLs.

Liver FABPs (L-FABPs) constitute 5% of hepatocyte cytosolic proteins and unlike other FABPs, L-FABP has two distinct binding sites with different affinities for FAs. The notion of how L-FABP discriminates between transporting LCFAs for lipoprotein synthesis and fatty oxidation implies interaction with other FATPs. L-FABP-null mice have diminished FA β-oxidation, indicating a major role in LCFA transport to mitochondria (Atshaves et al., 2010). The intestinal FABP (I-FABP) is expressed in the epithelium of the small intestine with L-FABP and ileal bile acid binding protein (IL-FABP), with each FABP showing regional distribution to

different segments of the intestine (i.e. L-FABP, proximal; IL-FABP, distal; and I-FABP, throughout). I-FABP-null mice have an enlarged liver and have weight gain, which suggests that L-FABP and G-FABPG may compensate for loss of I-FABP. L-FABP has a unique role in the intestinal synthesis of chylomicrons, which cannot be replaced by I-FABP. L-FABP seems to function in partitioning LCFA to PL biosynthesis, while I-FABP functions to partition LCFA to TAG synthesis. I-FABP has been linked to MetS through a mutation that increases postprandial serum lipids in humans (Furuhashi & Hotamisligil, 2008).

The heart and skeletal FABPs (H-FABP) function to direct acyl-CoA to mitochondria for FA β-oxidation. H-FABP-null mice show a switch from FA to glucose oxidation similar to the metabolic changes in the heart in ischemia, indicating that H-FABP is required for LCFA transport to maintain mitochondrial FA β-oxidation. H-FABP-null mice also display alteration in TG and PL with an increase in PA and decrease in AA (C20:4n6) incorporation in TG and PL. Both H-FABP and L-FABP regulate LCFA oxidation by activating peroxisome proliferator activated receptor (PPAR)-α. The adipocyte FABP (A-FABP, FAB4-aP2) transcription is controlled by FA, PPARγ, and insulin. A-FABP binds and activates hormone-sensitive lipase (HSL). A-FABP-null mice are protected from atherosclerosis, suggesting a role in MetS. It has been suggested that A-FABP has a central role in foam cell formation in macrophage through suppression of PPARγ-liver-X-receptor (LXR)-α activation of ATP-binding cassette (ABCA1)-mediated cholesterol efflux, induction of inflammatory cytokines, iNOS, and cyclooxygenase 2 (COX 2). The induction of A-FABP in intestinal epithelial cells by Th2 cytokines, interleukin (IL)-4 and IL13, mediated through GPR84 increase T and B cell IL4 secretion and links FA transport and immune response. It will be of interest to determine if the FA receptor (GRP84) and A-FABP are coordinately regulated during MetS.

Epidermal FABP (E-FABP) has a similar FA affinity and reactivity as adipocyte A-FABP. Adipocytes E-FABP-null mice have increased insulin-dependent glucose transport in adipocytes and may function with GPR40 in amplification of glucose-stimulated insulin secretion (GSIS). E-FABP binds retinoic acid and delivers to PPARβ for nuclear hormone receptor (NHR) activation (Schug et al., 2007). The skin type keratinocyte K-FABP functions in brain development where it provides a continuous supply of AA and DHA for neuron growth and axon development in membrane biogenesis. Brain B-FABP is expressed in glia of the white matter and strongly binds ω3-PUFA, and B-FABP-null mice have altered emotional responses typical

of schizophrenia. Neuronal FABP (N-FABP) is expressed in the peripheral nervous system where it maintains the lipid composition of myelin.

All FABP-knockout mice exhibit an increase in serum FFAs that are associated with obesity, diabetes and insulin, suggesting that the distribution of FAs to different cells rather than serum levels initiates MetS. In the A-FABP and E-FABP double knockout mice, serum FA composition shifts to short-chain FA (SCFA) with an increase insulin-stimulated glucose uptake, FA oxidation and AMP protein kinase (AMPK) activity. It is likely that serum saturated fatty acids (SFAs) activate adipocyte GPR41 and stimulate leptin release. Two other transporters of FAs are the plasma membrane (FABPpm) and FA translocase FAT/CD36. FABPpm has a unique amino acid sequence, which is identical to that of mitochondrial aspartate aminotransferase, a serum enzyme used to assess organ damage. Muscle contraction and AMPK activity increase the translocation of FABPpm to the plasma membrane in adipocytes and muscle cells. FAT/CD36 is a scavenger receptor protein with multiple functions in metabolic diseases, inflammation, and lipid metabolism. Expression of FAT/CD36 in muscle, adipocytes, and heart is critical for FA oxidation and esterification as revealed in FAT/CD36 knockout mice where muscle contraction was lost. It has been proposed that FAT/CD36 interacts with FATP and FABPpm to mediate FA transport across the lipid bilayer where FABP may facilitate absorption from inner plasma membrane leaflet. Insulin-Insig signaling pathway increases FAT/CD36 translocation to plasma membrane and association with FATP. Upon FA binding, FATP translocates to mitochondria and associates with carnitine palmitoyltransferase (CPT)-I, leading to oxidation of LCFA.

Unlike the close relationship between FATP and long-chain acyl-CoA synthetase (ACSL) where FATP facilitates FA uptake, while ACSL medicates activation, trapping, and vectorial acylation, FABP imports and transports FFAs to different organelles. The 10 organ-specific FABPs show difference in ligand selectively, binding affinity, and the mechanism of ligand binding. Unlike FATPs that solely transport FA-CoAs, FABPs show a broad range of ligands ranging from LCFA, lysoPLs, heme, and cholesterol. Organ-specific FABPs are largely overexpressed in both NAFLD and nonalcoholic steatohepatitis (NASH). Adipocyte FABPa is overexpressed in the liver and found at elevated levels in the serum of patients with NAFLD (Higuchi et al., 2011; Hoo et al., 2012; Kim, et al., 2011). Knockdown of A-FABP in Kupffer cells of mice fed a high-fat (HF) diet and administered LPS leads to resistance to steatohepatitis and hepatic production of proinflammatory cytokines (Higuchi et al., 2011; Hoo et al., 2012; Kim, Cho, et al., 2011).

In human patients with NASH, visceral adipose A-FABP levels are significantly higher than in subcutaneous adipose tissue, suggesting that either A-FABP from infiltrating macrophages or visceral adipocytes predisposes patients to progress from NAFLD to NASH. In patients with NAFLD, the level of L-FABP strongly correlates with the extent of obesity and levels of hepatic lipid accumulation (Yoon et al., 2012). The prevention of liver damage in mice fed an HF diet and administered LPS to induce acute liver injury by treatment with an A-FABP inhibitor suggests that targeting the adipocyte-monocyte A-FABP may be a novel therapy to prevent NAFLD progression to NASH (Higuchi et al., 2011).

2.1.3. ACBP-acyl-CoA Binding Protein

ACBPs are multifunctional housekeeping proteins that show tissue-specific distribution similar to FATPs and FABPs and are responsive to glucose and insulin signaling. There are four members of ACBP protein, which all bind C14–C22 acyl-CoA esters with high affinity ($K_d = 1$ nm) and specificity. ACBPs inhibit acyl-CoA ester hydrolysis and provide acyl-CoA esters to PL, glycerolipid, cholesterol ester, and ceramide synthesis. ACBPs prevent acyl-CoA inhibition of several metabolic enzymes, including acetyl-CoA carboxylase (ACC), ACSL, CPT1, adenine nucleotide translocator and acyl-CoA: cholesterol acyltransferase. Proteolytic fragments of ACBP have been shown to inhibit benzodiazepine receptors that are found at high levels in lymphocytes, macrophages, platelets and granulocytes. Furthermore, ACBPs function in FA metabolism, steroid synthesis and in the regulation of insulin secretion, cholecystokinin secretion, inflammation, and apoptosis. There are four ACBPs in humans that have been extensively characterized: those in liver, testis, brain and recently identified adipose ACBPe (Ludewig et al., 2011). Recently, additional 10 variant human ACBPs have been identified and shown to be transcriptionally controlled by sterol regulatory element binding protein 2 (SREBP2), hepatocyte nuclear factor 4α (HNF4α) and NF-κβ that function as central regulators of cholesterol, glucose metabolism and inflammation (Nitz, Kruse, Klapper, & Doring, 2011). Because ACBPs have very high affinities for plasma membrane, it has been proposed that ACBPs are responsible for PL turnover. Depletion of ACBP in cells increases short-chain unsaturated FA in the membrane PI and PC and reduces SFAs. ACBP has recently been shown to function in maturation of SREBP through activation of SREBP cleavage-activating protein in ER and insulin-induced gene (Insig) pathway. It is not known whether membrane structure or steroid levels prevent maturation of

precursor SREBP. Additional studies need to be performed to determine if ACBPs are able to transport PUFAs and possibly eicosanoids (Ludewig et al., 2011).

The role of ACBPs in MetS and NAFLD unlike other FATPs has not been as extensively studied, even though they distribute FA-acyl-CoA to different organelles and have intrinsic responsiveness to glucose and insulin. Furthermore, ACBPs are regulated by transcriptional factors that control fat and glucose metabolism (HNF4α, SREBP) and inflammation (NF-κβ) and regulate the partitioning of FAs between esterification and β-oxidation pathways (Nitz et al., 2011). Transgenic mice overexpressing ACBP show increased accumulation of different lipid classes and increase in liver TAG, suggesting that they have an important role in hepatic steatosis (Huang et al., 2005). The importance of ACBP in hepatic steatosis is further revealed in ACBP- knockout mice that show a delayed induction of lipogenic and cholesterogenic gene pathways due to a decrease in proteolytic processing of SREBP1 and SREBP2 (Neess et al., 2011). Because ACBP and acyl-CoA levels are similar in cells, it is not known whether increased acyl-CoA levels would occur in ACBP knockout mice and lead to increase in peroxisomal, microsomal, and mitochondrial FA oxidation. However, increased acyl-CoA content correlates with muscle insulin resistance but not liver insulin resistance, indicating that excess acyl-CoA has tissue-specific effects in the regulation of metabolism and the prevention of acyl-CoA lipotoxicity. Increased acyl-CoA levels have a dramatic effect on metabolism by covalent and allosteric enzyme modification as well as transcriptional mechanism dependent on FA or acyl-CoA chain length and degree of unsaturation. An imbalance in the FA and acyl-CoA pools would also influence β-oxidation and the synthesis of complex lipids, PLs, cholesterol esters, ceramide, and triglycerides.

2.1.4. Cellular Uptake of FFAs

Macrophages express both A-FABP and E-FABP that have distinct functions in lipid metabolism (Storch & Thumser, 2010). Macrophage-specific A-FABP−/−ApoE−/− congenic mice are protected from diet-induced obesity (DIO) and atherosclerosis (Erbay et al., 2009), possibly through A-FABP-mediated cytokines, which activate c-Jun-N-terminal kinase (JNK1). JNK1 has been shown to increase expression of A-FABP, which binds PPARβ and delivers FA ligand to PPARs. FATP and FABP are closely linked to metabolic inflammatory process through their ability to supply FA to critical lipid metabolic pathways for TG, PL synthesis, FA oxidation, and

recently as transporter of key LCFA to the nuclear receptor (NR) to modulate both metabolic and inflammatory transcription pathways. It is rather unfortunate that there are no studies on the role of FATPs or FABP in eicosanoid transport, although a number of studies have been performed on the transport of SFAs and uSFAs of different lengths. The expression of similar FABPs in adipocytes and macrophages indicates a link between the energy needs for inflammation and the maintenance of metabolic homeostasis where disruption of this balance initiates an unintended immune response leading to MetS. The development of targeted inhibitors of A-FABP for LCFA transport, similar to knockout mice, displayed enhanced insulin sensitivity, reduced hepatic steatosis and atherosclerosis as well as a reduction in obesity-associated inflammatory cytokines through attenuation of JNK1 activity (Furuhashi et al., 2007). In the human population, a gene variant of A-FABP gene with reduced function protects against MetS (Tuncman et al., 2006). Similarly, reduced activity of FATP4 has a beneficial effect in MetS (Gertow et al., 2004).

2.1.5. ACBP and FABP Fatty Acid Transport to the Nucleus

Several FABPs have the helix-turn-helix domain critical amino acid residues for protein–protein and protein–membrane interaction as well as a nuclear localization region that allows FABPs to transport FAs to nuclear transcription factors. ACBP exerts direct effects on gene regulation through FA acylation of histone 3, which is dependent on CoA ester and reactivity of cysteine (Wilson et al., 2011). Many in vitro studies have shown that FABP and ACBP can bind NHR, PPARα and HNF4α and elicit a transcriptional response by providing an endogenous ligand, either LCFA or LCFA-CoA. Fatty acid NHR ligands in the nucleus have high affinity binding (K_ds in nanomolars) for NHRs and the ability to induce conformational changes in NHRs, resulting in coregulator recruitment to NHRs (Atshaves et al., 2010; Schroeder et al., 2008). Support for the role of LCFA-CoA as NHR ligand is seen in peroxisome acyl-CoA oxidase (ACOXI)-null mice with the hyperactivation of PPARα that leads to very-long-chain fatty acid (VLCFA) and VLCFA-CoA accumulation and increased FA β-oxidation. In adrenoleukodystrophy patients, VLCFA accumulates in the cytosol and there is no formation of VLCFA-CoA or hyperactivation of PPARα (Sanders et al., 2006). PPARα has a high affinity for polyunsaturated LCFAs, LCFA-CoAs and VLCFA-CoAs, but not saturated LCFAs or VLCFAs (Schroeder et al., 2008).

One controversial issue concerning eicosanoids as in vivo activators of PPAR is whether micromolar concentrations of PGs, LTs, and cytochrome P450 eicosanoid metabolites for receptor activation can be reached in the nucleus. The transport of eicosanoids by FABP and ACBP may be one mechanism to reach these local HNR-activating concentrations. In contrast to PPARα, HNF4α have high affinity for saturated LCFA and VLCFA acyl-CoA but not polyunsaturated acyl-CoA, suggesting that FA-CoA chain length and degree of unsaturation determines whether HNF4α or PPARα will be activated (Hostetler et al., 2006). FABP binds PUFAs with greater affinity than saturated LCFA and activates PPARα, while ACBP preferentially binds saturated LCFA and activates HNF4α (Schroeder et al., 2008). Recently, HNF4α has been shown to bind linoleic acid (C18:2n6); however, receptor activation was not observed (Yuan, Ta, et al., 2009), suggesting that unsaturated LCFA may inhibit HNF4α activity. These results also suggest that ACBP selectivity cooperates with HNF4α, while L-FABP selectively cooperates with PPARα to mediate downstream coactivator or corepressor association with HNR. Thus saturated LCFA binding to HNF4α would increase activity and inhibit PPARα transactivation, while polyunsaturated LCFA-CoA would decrease HNF4α activity and increase FABP-PPARα transactivation. Because PPARα and HNF4α regulate transcription through a similar direct repeat 1 sequence and compete for the same coactivators and corepressors, receptor activation would be determined by saturated or polyunsaturated LCFA ligands, while crosstalk between these receptors would be determined by FABP/ACBP-mediated coregulator recruitment and cognate receptor repression (http://www.CISREG.ca/tfe).

Future studies need to address the in vivo role of FABP and ACBP protein in the transport of eicosanoids to the nucleus by immunohistochemical colocalization of labeled LCFA ligands, transport protein (FABP, ACBP) and NR (PPAR, HNF4) with receptor-mediated gene activation. Indeed, transgenic mice overexpressing ACBP fed an HF diet have ACBP-induced tissue-specific regulated expression of PPARs and SREBP (Oikari et al., 2008). In contrast, L-FABP gene ablation inhibits PPARα transcription of genes coding for LCFA FA oxidation (Atshaves et al., 2010).

The large number of FA transporters, FATP, FABP, ACBP, and ACSL, in vectorial acylation begs the question why there are so many transporters. Fatty acids have multiple roles in the synthesis of triglycerides, PLs, and eicosanoids, as well as catabolism for energy production, therefore the selective uptake and channeling to different organelles is essential for cell

survival. The variety of FA cellular transporters function to control uptake of FA when extracellular FA concentrations are low and to prevent lipid toxicity when extracellular concentrations are high, thus serving as a thermostat in regulating the metabolic needs of the cell in a changing environment. A prevalent view is that passive diffusion and protein-mediated transport contribute to FA uptake. The current concept of FA transport suggests that carboxylated FAs bind to basic residues of caveolin-1 and partitions into the plasma membrane and then internally diffuse to lipid rafts before reabsorption. Membrane proteins function to absorb FA from extracellular media, modulate transport across membrane, trap them intracellulary, and channel FAs to organelles dependent on FA chain length, degree of unsaturation, and metabolic needs.

In recent years, a number of proteins that facilitate FA transport in mammalian cells have been identified. These proteins include CD36/FAT, FABP, FATPS, and ACBP. Although these proteins have different tissue expression patterns, subcellular localization, and FA chain length and unsaturation specificity, each transporter acts independently to modulate FA transport to cellular needs and prevents marked elevation of free FA concentration that can lead to lipid toxicity and cell death. It is apparent that the level of cellular CoA and FABP binding of FFAs is necessary to prevent lipid toxicity.

2.2. Acyl-CoA Synthetase Channeling of FAs

The first step for FAs to be incorporated into cellular PLs is the thioesterification with CoA. The ACSVL/FATP family of ACSL consists of five members, ACSL 1, 2, 4, 5 and 6 with ability to channel FA to different organelles (Watkins et al., 2007). ACSL members show selectivity in the esterification of LCFA depending on degree of unsaturation, with ACSL 3, 4 and 6 showing selectivity toward the thioesterification of arachidonic acid. Gain- and loss-of-function studies have suggested that individual ACSLs channel FA-CoA to different organelles. ACSL1 channels oleic acid toward DAG and PL synthesis and away from cholesterol esterification. ACSL 3 knockdown decreases oleic (C18:1n9) PL incorporation for very low density lipoprotein (VLDL) synthesis (Yao & Ye, 2008). Knockdown of ACSL3 in rat hepatocytes significantly decreased the activation of several lipogenic transcriptional factors, PPARγ, carbohydrate-responsive element binding protein (ChREBP), SREBPIc and LXRα and their target genes suggesting a role in the control of hepatic lipogenesis. ACSL3 associates with the membrane structure of LDs, providing activated FAs for the PC by lysophosphatidylcholine acyltransferase (LPCAT), which enables growth of PL monolayer to keep up with the expanding core

when TAG synthesis is high. ACSL3 has a marked preference for AA and EPA over other uSFAs. ACSL4 is predominately expressed in steroidogenic tissues and is localized in peroxisomes and mitochondria and CoA activates preferentially AA and EPA. ACSL5 is located in the outer mitochondrial membrane in intestine and liver and uses a wide range of both SFAs and uSFAs. ACSL5 overexpression in cell increases the incorporation of LCFA into LD for TAG synthesis (Bu & Mashek, 2010). In addition to different FA preference, tissue distribution, and organelle location, ACSLs are differentially regulated by pharmacological inhibitors such thiazolidinedione (TZD).

2.3. Synthesis of Triacylglycerol

Fatty acid transport and channeling have a vital role in the TAG and PL synthesis with regard to lipid metabolism and eicosanoid synthesis, respectively. The synthesis of TAG is initiated by the acylation of glycerol-3-phosphate by several glycerol phosphate acyl transferase (GPAT) isoforms. An extensive review on TAG synthesis has been recently published and the reader is referred to this article for further information (Coleman & Mashek, 2011). We will briefly summarize the steps of TAG and PL synthesis to give the reader a contextual framework to understand their importance in release of eicosanoid precursors by phospholipase A_2 enzymes. There are four GPATs with GPAT1 being the major GPAT in the liver and has a preference for C16:0. In GPAT(−/−)−/− mice there is a reduction of palmitic acid at the sn-1 position with an increase in sn-2 AA in PC, PE and PI, suggesting that the sn-1 FA determines the FA incorporated into sn-2 position. GPAT1 location in the outer mitochondrial membrane competes with CPT1 for FA since overexpression of GPAT decreases FA β-oxidation while increasing DAG hepatic content. GPAT also appears to function in the inflammatory response since GPAT1-null mice have an increased abundance of proinflammatory cytokines due to an increase in PGE_2 and LTB_4 resulting from an increase in hepatic and lymphocyte AA pools (Collison et al., 2008; Karlsson et al., 2009). This is reminiscent of a chronic inflammatory response seen in many diseases of MetS. GPAT2 is a second mitochondrial isoform but unlike GPAT1 has no FA preference. GPAT3 is the first ER GPAT and shows a high preference for C12:0–CoA and is prominently expressed in adipocytes (Kim et al., 2010). GPAT4 is expressed in liver and adipose tissue. GPAT4-null mice show a 50% decrease in hepatic TAG synthesis and increased energy expenditure and are resistant to fatty liver (Nagle et al., 2008).

GPAT-generated lysophosphatidic acid (LPA) is converted to PA by different sn-2-acylglycerol-3-phosphate acyltransferase (AGPAT) located in ER

and mitochondria. AGPAT1 incorporates AA and/or stearic-CoAs into the sn-2 when palmitic acid is in the sn-1 position. AGPAT2 is expressed in liver, heart and adipocytes and has a role in adipocyte differentiation since deficiency causes human lipodystrophy. AGPAT3 has high activity toward acylation of lysophosphoinositol and its overexpression increases PL species containing PUFAs. Although all AGPAT isoforms incorporate uSFAs in the sn-2 position, their activities for different lysoPL and type of uSFA remains unclear.

The final step in the synthesis of TAG is the hydrolysis of PA by sn-3-phosphatide phosphohydrolase (PAP/LP) of the lipin family located in the plasma membrane. Other lipin members translocate from ER to cytosol to hydrolyze PA formed by AGPAT to produce DAG that can either be converted to TAG or used for synthesis of PLs by the Kennedy pathway. There are three members of PAP lipin family that contain a nuclear localization sequence that interacts with transcription coactivators. Lipin1 interacts with PPARα, HNF4α, and peroxisome proliferator activated receptor coactivator (PGC)-1α to promote transcription of FABP4 and cytokine expression (Kim et al., 2010). Lipin1 has the highest PAP hydrolase activity of all lipins, yet all lipins have similar coactivator activities (Csaki & Reue, 2010).

PAP hydrolase produces 1, 2-diacylglycerol (DAG) that is converted to TAG by diacylglycerol acyltransferase 1 or 2 (DGAT1) located in ER, mitochondria and LDs. Neither DGAT1 nor DGAT2 has a preference for acyl chain length or degree of FA unsaturation. It is unclear whether these DGATs synthesize different TAGs in LDs or in VLDL synthesis. However, overexpression of either DGAT increases LD formation and steatosis, while DGAT1-null mice are resistant to obesity or steatosis when fed an HF diet (Smith et al., 2000).

The synthesis of DAG from PA by lipin is followed by TAG or PL synthesis. How a cell determines whether to synthesize TAG or PL seems to depend on the ATP levels, with high ATP favoring PL synthesis, while low ATP levels favoring increased TAG synthesis (Wu & Carman, 1994). For PL synthesis, different bases are added to the sn-3 position to produce PC, PE, PS, and PI. The free AA cell pool is controlled by PLA_2-mediated cleavage of sn-2 position of PL to produce a free FA and lysoPL and a CoA-dependent acyltransferase acylation reaction reincorporates a different uSFA-CoA to reform the PL. In stimulated cells, PLA_2-mediated deacylation is the dominant reaction, while in resting cells, the reacylation reaction dominates. In stimulated cells, the released AA is used for eicosanoid synthesis, while the lysoPL is reacylated by lysophospholipid: acyltransferase (LPLAT) a member of the membrane-bound O-acyltransferase family that uses FA-CoA for incorporation. The LPLATs that show preference for reacylation of lysoPL

include lysoPC:acyl-CoA acyltransferase 2 and 3 as well as lysoPI:acyl-CoA acyltransferase and lysophosphatidic acid: acyl-CoA acyltransferase 3. Of particular interest is that the LD lipase CGI-58 has lysophosphatidic acid:acyltransferase activity has a high preference for AA (Moessinger et al., 2011; Shindou et al., 2009) PLs are in constant state of remodeling through the Land's cycle by the action of PLA_2 and lysophospholipid:acyltransfera se (LPLAT) that use CoA-independent FFAs as substrate. The cellular AA pool is in constant flux and is determined by PL deacylation by PLA_2 and reacylation by lysophospholipid acyltransferase (LPLAT). Thus there are two biosynthetic pathways for the incorporation of AA-CoA into PL. The Land's cycle transacylates lysoPL to ensure the proper distribution of FAs to produce numerous cellular PLs, and the Kennedy pathway for synthesis of lysoPC from PA incorporates AA-CoA in the sn-2 position by lysoPL-CoA-dependent acyltransferase (LPLAT).

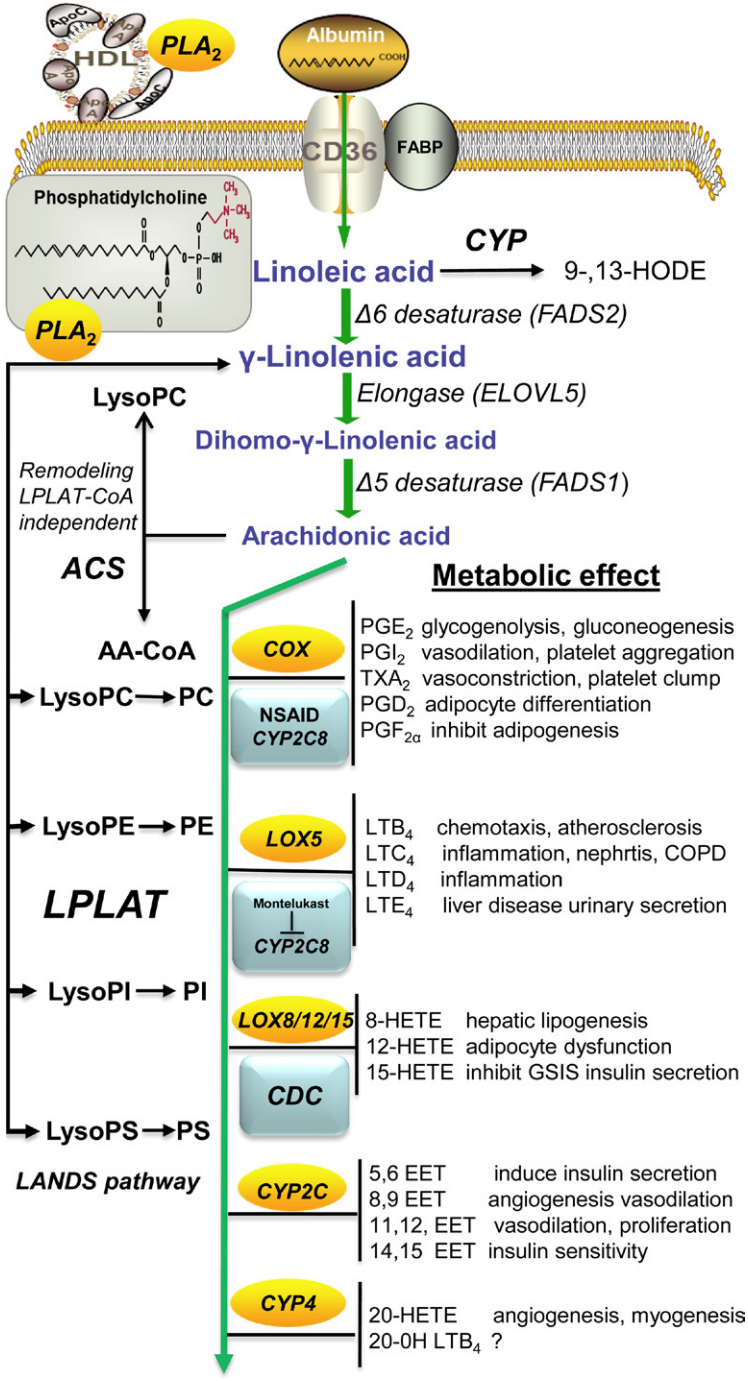
The products of TAG and PL metabolic pathways affect several cellular signaling pathways involved in MetS. Increased TAG content in the liver, pancreas and muscle is strongly correlated with insulin resistance. There are four currently known lipid metabolites that may account for insulin resistance, FA-CoA, ceramide, DAG and oxidized lipids. Lipid overload as the cause of insulin resistance is evident from the overexpression of lipoprotein lipase that increases tissue TAG, DAG, acyl-CoA and ceramide, while inhibition of adipocyte lipolysis in diabetic patients decreases muscle acyl-CoA and insulin resistance (Bajaj et al., 2005). The paradox of insulin signaling in insulin resistance is how the phosphorylation of insulin receptor (IR) substrate-1 (IRS-1) increases lipogenesis, but phosphorylation of IRS-2, which normally inhibits gluconeogenesis by FOXO1 phosphorylation and exclusion from the nucleus, does not inhibit liver gluconeogenesis (Brown & Goldstein, 2008). It is believed that a lipid metabolite inhibits IRS-2 phosphorylation of FOXO1 and thus prevents inhibition of gluconeogenesis. Strong support for lipid metabolites causing insulin resistance comes from studies of knockout and overexpression of TAG biosynthetic enzymes. Overexpression of GPAT1 in mice causes an increase in DAG activation of protein kinase C (PKC)-ε, leading to insulin resistance with no sign of inflammation, indicating a dissociation of insulin resistance from inflammation (Nagle et al., 2009). In contrast, GPAT1-null mice have a lower hepatic content of DAG and PKCε activation with a twofold increase in acyl-CoA and show reduced steatosis and insulin resistance (Li et al., 2010). Therefore, if DGAT1 activity is associated with insulin resistance through DAG activation of PKCε and phosphorylation and inhibition of IRS-2, then overexpression of DGAT should increase insulin

resistance. However, overexpression of DGAT1 or DGAT2, which increased hepatic content of TAG, DAG, ceramide and acyl-CoA, did not show insulin resistance or inflammation. Thus, it is possible that overexpression of GPAT causes insulin resistance, while DGAT overexpression protects against insulin resistance, suggesting that possibly LPA promotes, while PA prevents insulin resistance. Adipose triglyceride lipase (ATGL)-null mice have increased muscle DAG levels, yet are glucose tolerant and insulin sensitive, suggesting that PA or DAG protects against insulin resistance.

The tenet that DAG activation of PKCε-mediated serine phosphorylation of IRS-2 causes insulin resistance is questioned by studies using lipin (phoshatidic acid hydroylase)-null mice that inhibit the formation of DAG from PA. Mutation of Lipin1 gene in fatty liver dystrophy (fld) mice leads to lipodystrophy and insulin resistance, while Lipin1 overexpression leads to obesity. In obese human patients, reduced lipin1 levels were found in insulin-resistant patients. This correlation between high lipin and glucose tolerance and/or low lipin and insulin resistance was observed in other human studies. LIPIN1 polymorphisms are associated with insulin levels, body mass index (BMI) and risk of MetS, suggesting that lipid metabolites between GPAT and Lipin may be responsible for MetS insulin resistance. The numerous PL species produced by the Kennedy pathway and the Land's cycle PL remodeling pathway may have important roles in diabetes, obesity and MetS. Unfortunately, the generation of lysophospholipid: CoA acyltransferase (LPCAT) knockout mice have been lacking except for the recent report of LPCAT3 (lysoPC:acyl-CoA acyltransferase) liver-null mice that showed increased levels of lysoPC that promote VLDL by enhancing microsome transfer protein (MTP) expression and thus hepatic TAG accumulation. However, whether these mice have insulin resistance was not determined.

2.4. Desaturation of Unsaturated Fatty Acid in Eicosanoid Synthesis

The daily uptake of AA from western diets is calculated to be 0.3–2.0 g/day, while the intake of linoleic acid C18:2n6 is from 10 to 20 g/day and ALA C18:3n3 intake is between 2 and 5 g/day, indicating that the intracellular AA pool is largely determined by our diet. This pool of pro-inflammatory n-6 AA can be modified by reducing consumption of ω-6 linoleic acid and increasing consumption of n-3 linoleic acid. Both, linoleic and ALA are sequentially desaturated and elongated to produce AA and EPA, respectively (Fig. 5.2). The human FA desaturases are encoded by three


HDL
PLA2
Albumin
COOH
CD36
FABP
Phosphatidylcholine
PLA2
CYP
Linoleic acid
9-,13-HODE
Δ6 desaturase (FADS2)
γ-Linolenic acid
Elongase (ELOVL5)
Dihomo-γ-Linolenic acid
Δ5 desaturase (FADS1)
Arachidonic acid
LysoPC
Remodeling
LPLAT-CoA
independent
ACS
AA-CoA
LysoPC → PC
LysoPE → PE
LPLAT
LysoPI → PI
LysoPS → PS
LANDS pathway
Metabolic effect
COX
PGE2 glycogenolysis, gluconeogenesis
PGI2 vasodilation, platelet aggregation
TXA2 vasoconstriction, platelet clump
PGD2 adipocyte differentiation
PGF2α inhibit adipogenesis
NSAID
CYP2C8
LOX5
LTB4 chemotaxis, atherosclerosis
LTC4 inflammation, nephrtis, COPD
LTD4 inflammation
LTE4 liver disease urinary secretion
Montelukast
CYP2C8
LOX8/12/15
8-HETE hepatic lipogenesis
12-HETE adipocyte dysfunction
15-HETE inhibit GSIS insulin secretion
CDC
CYP2C
5,6 EET induce insulin secretion
8,9 EET angiogenesis vasodilation
11,12, EET vasodilation, proliferation
14,15 EET insulin sensitivity
CYP4
20-HETE angiogenesis, myogenesis
20-0H LTB4 ?

genes, FADS1, FADS2 and FADS3. FADS1 and FADS2 produce longer chain PUFAs by introduction of double bonds between specific carbons and elongation by Elongase (Elovl) enzymes to produce 20-carbon AA and EPA. Further metabolism of AA and EPA produces eicosanoids by the PG, LT and cytochrome P450 pathways.

FADS2 encodes a Δ6-desaturase, which is the first and rate-limiting step in the synthesis of ω-3 or ω-6 EPA and AA, respectively. Deletion of FADS2 abolishes the synthesis of PUFA and eicosanoids. FADS1 Δ5 desaturase

Figure 5.2 ***Metabolism of linoleic acid to proinflammatory eicosanoids and there function in control of metabolism.*** Arachidonic acid (AA) is obtained either directly from diet or synthesis from linoleic acid. This existence of PLA_2 in serum lipoproteins provides local release of arachidonic acid from membrane phospholipids and a source of eicosanoids to control inflammation, immune cell function and tissue-specific control of metabolism. Linoleic acid is converted to arachidonic acid by a series of fatty acid desaturase (FADS) and elongase (ELVOL) to produce AA. In humans, there is approximately 100 g of AA distributed between tissue and membrane compartment that have varying turnover rate depending on the metabolic needs of the tissue. Phospholipid AA incorporation occurs through the de novo Kennedy pathway or the Land's membrane remodeling pathway that requires AA-CoA. The remodeling of membrane PL can occur by the Lands pathway that uses lysophospholipid:acyl-CoA acyltransferase (LPLAT) or a CoA-independent transacylase pathway. AA released from membrane phospholipids can be used either to remodel phospholipid membranes or to synthesize eicosanoids, by the prostaglandin, leukotriene, cytochrome P4502 epoxygenase (CYP2), or the cytochrome P450 ω P4504 (CYP4) pathways. The biologically potent autocoid lipids of the series-2 prostanoids and series-4 leukotrienes initiate their biological effects by generally activating prostanoid and leukotriene receptors to initiate a proinflammatory response and activation of nuclear hormone receptor (NHR) and anti-inflammatory response. The CYP2 epoxygenase, epoxyeicosatrienoic acids (EET) also initiate their biological responses through presently unidentified membrane receptors and activation of peroxisome proliferator activated receptors (PPARs). In contrast, the CYP4 ω-hydroxylase metabolite's mechanism of action has not been identified. It is presently believed that 20-hydroxyeicosatetraenoic acid (20-HETE) directly interacts with membrane protein channels to elicit their potent vasculature constrictive and proinflammatory responses. Unlike our extensive understanding of eicosanoids' role in immune cell regulation during inflammation and their function in the cardiovascular system, our understanding of their role in the control of intermediary metabolism is lacking as revealed by our spare knowledge of their metabolic effects. By the use of drugs that inhibit eicosanoid synthesis, including the nonsteroidal antiinflammatory drugs (NSAID), we are able to modulate the cardinal signs of inflammation, pain, heat, redness, edema, and loss of function through possible metabolism by CYP2 epoxygenase resulting in reduced synthesis of EETs and channeling of AA to different eicosanoid metabolic pathways. CYP2C8 metabolizes many of the currently prescribed NSAIDs, and Montelukast inhibits CYP2C8, while CDC, a metabolite of ciproflazacin, inhibits 12/15-lipoxygenase. (For color version of this fi gure, the reader is referred to the online version of this book).

activity is 10-fold less than that of FADS2 and produces C20:3 and C20:4 PUFAs that are metabolized to less-active eicosanoids (Fan et al., 2012). FADS-null mice display reduced intestinal crypt proliferation, immune cell homeostasis, and heightened sensitivity to inflammation. The inability of FADS1-null mice to tolerate an intestinal inflammatory challenge is similar to that of the PGE-synthase-null and COX2-null mice (Nakanishi et al., 2011) where PGE_2 has a protective role in the intestines. FADS3 is a third gene identified in the FADS cluster that displays a unique expression in other organs different from liver expression of FADS1 and FADS2. Presently, the substrates for FADS3 have not been identified, although its upregulation during oxidative stress suggests a role in the prevention of lipotoxicity. FADS1 and FADS2 expression is increased by insulin, while FADS3 expression is mediated by PPARγ (Arbo et al., 2011; Reardon et al., 2012). There is a strong association of FADS2 gene with plasma liver enzyme levels, MetS, and type II diabetes mellitus (T2DM) (Chambers et al., 2011; Sergeant et al., 2012) and a negative association with FADS1. FADS1 and 2, Δ desaturases, activities require cytochrome b_5 and cytochrome b_5 reductase that are also used by stearoyl-CoA desaturase (SCD-1) that converts steric and palmitic acids to oleic and palmitoleic acid, respectively. Cytochrome b_5 also has an important role in the function and activity of cytochrome P450-mediated drug metabolism since Cyb_5-null mice have a 84% decrease in γ-linolenic (GLA) and 200% increase in alpha-linolenic acid (ALA). Cytochrome b_5 and cytochrome b_5 reductase (Cyb_5A, Cyb_5R3) or oxidoreductase supplies electrons for desaturase reactions. Cyb_5-null mice have impaired desaturation of palmitic and stearic acids and display lipotrophy with increased cytotoxic effects of SFAs. Both Cyb_5A and Cyb_5R3 reductase genes have been linked to obesity and diabetes susceptibility.

2.5. Elongation of PUFA in Eicosanoid Synthesis

The desaturation of linoleic acid (LA) and ALA by FADS2 produces GLA and stearidonic acids, respectively, which are elongated by elongase 5 (Elovl5). The Elovl family consists of six members in mouse and humans and carries out substrate-specific elongation of FAs of different lengths. The unsaturated VLCFA is elongated by ElovI, 3, 6, and elongation of PUFA is performed by ElovI 2, 4, 5. Elovl1 is involved in membrane modeling and Elovl2 elongates the PUFA, AA, EPA, DHA and docosapentaenoic acid (DPA) and has overlapping function with Elovl5. Elovl3 is found in brown adipose tissue (BAT) and Elovl3-null mice have impaired skin barrier function, complete

depletion of fat droplets, and cold intolerance. Elov5 is involved in the elongation C18-C20 unsaturated FAs but not PUFA longer than C22 to produce docosapentanoic acid (C22:5n-6). Elovl3 is found in peroxisomes and produces C24:4n6 and C24:5n3 FAs that are shortened to DPA and DHA. Elovl5-null mice have decreased elongation of 16:1ω7, and Elovl6-null mice show decreased elongation of both C16:1n7 and (Green & Olson, 2011). Both AA and DHA suppress SREBP and its target genes in FA triglyceride synthesis and development of hepatic steatosis (Moon et al., 2009). Elovl4 is expressed in retina, brain and testes and is involved in elongation steps required for DHA synthesis (Yu et al., 2012). Elovl5 induces changes in hepatic FA content and influences multiple pathways in lipid and carbohydrate metabolism and attenuates hyperglycemia in DIO mice and restores insulin sensitivity. Elov6 is involved in the elongation of C12-18 saturated FAs and is found in major metabolic tissues, BAT, WAT, liver and brain. Different single nucleotide polymorphism (SNP) alleles in the human population that have been correlated with insulin sensitivity in human (Morcillo et al., 2011). Recently, a seventh Elovl7 has been identified that has a high activity toward C18:3n-3 (Naganuma et al., 2011). The proper elongation and desaturation of FAs are essential to maintaining lipid homeostasis since disruption of these processes can lead to MetS diseases.

2.6. Phospholipase A_2 Role in Formation of Bioactive Lipids in MetS

The incorporation of AA into membrane can occur by three mechanisms: (1) lysophosphatidic acid acyltransferase (LPAAT) synthesis of DAG for PL synthesis through the Kennedy pathway, (2) the CoA-dependent acylation of DAG-CDP choline by lysoPL acyltransferase (LPLAT) or the (3) CoA-independent transacylation by head-group-specific lysophospholipid acyltransferase (LPLAT) of Land's cycle. Membrane lipid PI represents an important cellular signaling molecule. The release of inositol phosphate by PLC is an important regulator of metabolism. Phospholipase-A_2-mediated release of AA and lysophosphatidylinositol (lysoPI) also have important roles in both eicosanoid synthesis and metabolic control. LysoPI can bind LysoPI receptors, while AA can be used for reacylation of membrane lipids or used for the production of eicosanoids.

There are a number of recent excellent resources on structure, function and therapeutic modulation of PLA_2 (Dennis, Cao, Hsu, Magrioti, & Kokotos, 2011). The multiple functions of PLA_2 in blood stream, gastrointestinal

system and intracellular location strongly not only implicate that these proteins serve as guardians against foreign insults by activation of inflammation but also suggest that they control basic physiological and metabolic processes.

Therefore the intent of this section is to discuss the role of those PLA_2 enzymes in MetS. There are 30 phospholipase A_2 and related enzymes that include the 11 secreted Ca^{2+}-requiring extracellular PLA_2 ($sPLA_2$), the six Ca^{2+}- dependent cytosolic PLA_2 ($cPLA_2$) also known as patatin-like phospholipase domain containing lipase (PNPLA) that function as either phospholipase or lipase, the nine Ca^{2+}-independent PLA_2 ($iPLA_2$), the two lysosomal PLA_2, the four platelet activating factor acetylhydrolases (PAF-AH) that have specificity for platelet activating factor (PAF) or oxidized PLs, and the adipose-specific PLA_2. Phospholipase A_2 can be largely divided into two groups: (1) those that function in immune response and inflammation such as, $cPLA_2$, PAF-AH, and sPLA2 and (2) those that function in metabolic disease such as $sPLA_2$, $iPLA_2$, lysosomal, and adipose PLA_2.

The secreted family of $sPLA_2$ has 11 members that have antibacterial and antiviral function as well as a role in diseases with an inflammatory etiology and are often referred to as the "inflammatory $sPLA_2$". These secreted $sPLA_2$ function through two independent pathways, the heparin sulfate proteoglycan (HSPG)-dependent and HSPG-independent pathways, to release AA for PL. Secreted $sPLA_2$ can bind to cell surface HSPG that internalizes the enzyme through caveolin-dependent endocytic pathway where it releases AA from internal anionic PL substrates. In the HSPG-independent pathway, PLA_2 binds to bacterial apoptotic cells and exosomes anionic PLs, PLE, PLG, PS, and PA to release AA. PLs entering the digestive tract through food intake or bile acid recycling as PL coated TAG vesicles hydrolyzed to FAs and lysoPLs by PLA_2, subsequently allowing digestion bo TAG by pancreatic lipase and carboxyl ester lipase for proper intestinal absorption. The $sPLA_2$IB is a pancreatic enzyme that digests dietary PLs. PLA_2IB-null mice are resistant to obesity and show reduced plasma levels of insulin, leptin and glucose on a diabetogenic diet due to reduced intestinal absorption of PL and increased FA β-oxidation by PPARα. Thus inhibition of PLA_2IB may be an ideal drug target in the treatment of obesity and diet-induced diabetes.

The relationship between secreted $sPLA_2$ and atherosclerosis is a major area of research since PLA_2 is able to modify plasma lipoproteins where upon PC hydrolysis it produces FFA and lysoPC that can trigger

vasoactive, chemoactive and proinflammatory conditions (Rosenson & Gelb, 2009). $sPLA_2$ mediates hydrolysis of LDL to small dense proatherogenic LDL particles, while hydrolysis of high-density lipoprotein (HDL) reduces cholesterol efflux from lipid-rich foam cells. Atherosclerotic lesions containing more lysoPC alter apo-B-100 conformation and promote particle aggregation (Rosenson & Gelb, 2009). Transgenic $sPLA_2$IIA mice have an increased incidence of atherosclerotic lesions, while macrophage $sPLA_2$ exerts a local proatherogenic effect independent of systemic lipoprotein metabolism (Ghesquiere et al., 2005). A hyperlipidemic HF diet increases $sPLA_2$V expression in aorta and induces atherosclerosis by assisting in macrophage uptake of LDL particles. Macrophage $sPLA_2$X is also able to hydrolyze LDL HDL and attenuates cholesterol efflux from macrophage. PLA_2X-null mice show an attenuated accumulation of neutrophils in ischemic areas of the heart due to reduced production of LTB_4, indicating that $sPLA_2$X has a direct role in neutrophil myocardial injury. It is apparent that $sPLA_2$IIA, $sPLA_2$V and $sPLA_2$X have a significant role in atherosclerosis and CVD; however, their roles in MetS are not fully understood.

Increased release of FFAs and LPC from circulating lipoproteins in diabetic individuals by PLA_2V not only increases an individual's risk of CVD but also contributes to MetS. Stress-induced increases in serum glucocorticoids are evident in MetS patients. In $sPLA_2$X-null mice, there is an 80% increase in plasma corticosterone levels, resulting in oxysterol activation of LXRα and macrophage ABCA1 cholesterol efflux transporter. Excessive glucocorticoids can lead to Cushing-like syndrome and associated lipodystrophy observed in obese individuals with MetS. Unfortunately, these lipid derangements associated with MetS have not been investigated in PLA_2X-null mice.

The intracellular $iPLA_2$ consists of six calcium-dependent $cPLA_2$ and nine Ca^{2+}-independent $iPLA_2$ that are expressed in many tissues. The most widely studied are these PLA_{2s} because they produce a diverse array of functional lipid products in response to extracellular stimuli. The cytosolic $cPLA_2$ has both phospholipase A_2 and lysophospholipase activity. It is the only PLA_2 that shows specificity for PLs containing AA exclusively. Thus the cytosolic $cPLA_2$ has a pivotal intracellular role in the production of eicosanoids and a functional role in normal physiological process and the disease pathology. Cytosolic $cPLA_2$IVA is activated by Ca^{2+}-dependent translocation from the cytosol to the perinuclear membrane and functionally couples with COX in PGE_2 biosynthesis. The preferred substrate for

cPLA$_2$IVA is phosphatidylinositol-4, 5-bisphosphate, which also fully activates cPLA$_2$IVA. Cytosolic cPLA$_2$IVA-null mice display a number of phenotypes due to reduced synthesis of eicosanoids in a broad range of tissues. The broad and extensive pathophysiological role of cPLA$_2$IVA revealed by cPLA$_2$IVA-null mice has been extensively documented (Murakami et al., 2011).

Cytosolic cPLA$_2$IVA-null mice are protected from MetS and associated atherosclerosis, obesity and hepatic steatosis, however its mechanistic role in these diseases has not been studied extensively. Cytosolic cPLA$_2$IVA is localized on LDs, which are present in all cell types and consist of a hydrophobic core of TAG and cholesterol esters surrounded by a monolayer of PLs and cholesterol. Cyotsolic cPLA$_2$IVA phosphorylation by JNK1 and ceramide kinase increases LD formation, while cPLA$_2$IVA knockdown inhibits LD formation (Gubern et al., 2008). Cytosolic cPLA$_2$IVA-null mice are protected from accumulation of LD in adipose tissue and liver (hepatic steatosis) under normal and HF diets. Thus these mice are refractory to atherosclerosis. Because cPLA$_2$IVA-null mice fed an HF diet have no difference in serum leptin, resistin, FFA, VLDL, glucose or insulin compared to the corresponding wild-type mice, it is suggested that cPL$_2$IVA might be an amenable drug target for NAFLD and other obesity-related diseases. The regulation and function of other cytosolic cPLA$_2$ isoforms are less well known with respect to substrate preference and physiological phenotype in disease processes. Cytosolic cPLA$_2$IVB displays a 1000-fold greater lysophospholipase activity compared with other PLA$_2$ (Ghomashchi et al., 2010) and associates with mitochondrial membrane phosphoinositide rich in cardiolipin (CL). Cytosolic cPLA$_2$IVC binds to heart mitochondrial membranes and ER where it is believed to function in membrane PL remodeling because of its high lysophospholipase and transacylase activity.

The human Ca^{2+}-independent iPLA$_2$ consists of nine members that are also known as PNPLA1-9. Patatin is a lipid hydrolase and therefore mammalian PNPLAs have specificity for diverse substrates, including TAG, PL and retinol esters. More than half of the enzymes in this family function as lipases rather than phospholipase. Several of the PNPLA members have important physiological roles in lipid metabolism and energy homeostasis, and thus MetS.

The classical independent iPLA$_2$VIA also known as PNPLA9 exhibits sn-1 lysophospholipase and transacylase activity and thus functions in membrane remodeling through the Land's cycle. It has a fundamental role in cell signaling leading to cell activation, proliferation and migration. Independent

iPLA$_2$VIA translocation from cytosol to membrane is mediated by PKC. In pancreatic β-cells, iPLA$_2$VIA overexpression enhances glucose-induced AA release and insulin secretion (Ma et al., 2001). Independent iPLA$_2$IVA functions in the regulation of capacitive Ca^{+2} entries with calmodulin, which inhibits iPLA$_2$IVA. Upon Ca^{+2} depletion, calmodulin dissociates from iPLA$_2$IVA, which generates lysoPL that opens Ca^{+2} entry (SOCE) channels with increased Ca^{2+}, which activates cPLA$_2$IVA-mediated release of AA. Thus cPLA$_2$IVA has an important functional role in Ca^{2+} homeostasis and eicosanoid synthesis in vascular contraction and relaxation (Xie et al., 2010). Disruption of iPLA$_2$IVA in the pancreas impairs insulin secretory response to glucose, and iPLA$_2$IV-null mice on an HF diet have severe glucose intolerance and coronary-artery-induced occlusion. Intracellular iPLA$_2$IVA, which hydrolyzes membrane PLs, induces lethal malignant ventricular tachyarrhythmia and myocyte apoptosis during acute cardiac ischemia. During apoptosis, cells release FAs and LPC, which are mediated by caspase 3 cleavage of iPLA$_2$IVA to a more active enzyme. The translocation of iPLA$_2$IVA during stress conditions to the mitochondria results in loss of mitochondrial PL and release of cytochrome c and opening of the mitochondrial permeability transition pore.

Independent iPLA$_2$IVA also plays an important role in MetS as revealed not only by impaired glucose-stimulated insulin secretion in iPLA$_2$IVA-null mice but also by the fact that these mice show age-related loss of bone mass with increase in bone marrow fat-laden adipocytes due to alteration in mesenchymal progenitor cells toward adipocytes and osteocyte lineages (Ramanadham et al., 2008). Overexpression of iPLA$_2$IVA increases TAG synthesis and LD formation, suggesting that iPLA2IVA provides FAs for TAG synthesis from the PL pool and also increases LD formation, thus recycling structural PLs for energy-generating substrates. Independent iPLA$_2$IVA has multiple roles at different stages of inflammation by production of lysoPC that attracts neutrophils and monocytes through lysoPC GPCRs, activation of NADPH oxidase in neutrophils during respiratory burst, and neutrophil phosphatidylserine (PS) externalization leading to induction of intrinsic apoptotic pathway (Lei et al., 2010).

Independent iPLA$_2$VIB (PNPLA8) is localized to mitochondria and peroxisome and cleaves PLs at the sn-1 and sn-2 positions depending on the substrate. Mice null for iPLA$_2$IVB are lean and resistant to adiposity, fatty liver, hyperlipidemia, and HF-diet-induced insulin resistance and glucose intolerance (Song et al., 2010); however, these mice display abdominal lipodystrophy and impaired insulin secretion on an HF diet. In skeletal muscle,

iPLA$_2$VIB-null mice have impaired mitochondrial β-oxidation as reflected by increased accumulation of long-chain acylcarnitine. In the heart, these knockout mice generate signaling lipid metabolites that modulate energy storage and utilization in different metabolic states by remodeling of CL and thus tailoring mitochondrial lipid composition and metabolism. Knockdown of iPLA$_2$IVB by siRNA reduces cytokine and chemokine overexpression, while overexpression leads to increased PGE$_2$ production through COX1 activation (Murakami et al., 2011). It is uncertain how reduction in selective eicosanoid metabolites in iPLA$_2$IVA-null mice relates to the lean phenotype and resistance to MetS. Data strongly support that iPLA$_2$IVA plays a role in integrating lipid and energy metabolism and possibly through inefficient coupling of electron transport to energy production promotes development of MetS.

Both PNPLA2 (ATGL) and PNPLA3 (adiponutrin) enzymes have attracted much interest in the past few years because of their roles in obesity and MetS. PNPLA2, also known as ATGL, possesses transacylase with weak PLA$_2$ activity and is recruited to LD during lipogenesis (Soni et al., 2009). A number of cofactors regulate PNPLA2/ATGL, which was discussed in an excellent review on ATGL's role in adipose lipolysis (Lass et al., 2011). PNPLA2-null mice display severe defects in TG hydrolysis leading to lipid accumulation in WAT and BAT, while overexpression promotes lipolysis and inhibits DIO. Several SNPs in PNPLA2 in type II diabetic patients are correlated with reduced plasma FA and TAG. PNPLA2-null macrophages fail to hydrolyze cellular TAGs, thus decreasing cellular levels of FAs, but with an accumulation of LDs. This results in decreased cellular ATP production and impairment of phagocytosis, suggesting that FA must go through a cycle of esterification and hydrolysis before it can be used for energy production (Ahmadian et al., 2009). This implies that FATP, FABP or selective ACSL must channel FA to TG or membrane PL prior to ATGL release and use as an energy substrate. A second TG hydrolyase is PNPLA3 also named adiponutrin, which is found in adipocytes and induced by insulin and in steatotic liver of ob/ob mice where it is induced 100-fold by through LXRα agonist activation of SREBP1 (Huang et al., 2010). PNPLA3 has TAG lipase and transacylase activities with weak PLA$_2$ activity. PNLA3 gene variants are associated with hepatic steatosis and liver function in NAFLD (Tian et al., 2010). A point mutation in PNPLA3 I148M disrupts TAG hydrolytic activity leading to hepatic steatosis (He et al., 2010). Both PNPLA2 and PNPLA3 are TAG hydrolases that modulate TAG content

in adipocytes and LD formation in liver and are genetically linked to obesity in humans.

The PAF-AH family members are unique acyl hydrolases that catalyze the release of acetate from the sn-2 position of platelet activity factor (1-0-alkyl-PC). There are four enzymes in this family that are associated with eicosanoid and possibly MetS. The plasma-type PAF-AH (PLA_2V11A) has attracted much attention recently in regard to a therapeutic target for atherosclerosis. PAF-AH hydrolyzes acetate or an acyl group up to nine carbons in length from the sn-2 position of PC or PE producing lysoPAF. PAF-AH is associated with apo-B-100 of LDL and HDL where it removes oxidized PC from LDL particles. PAF-AH expression is dramatically increased after LPS administration in several tissues, including circulating leukocytes where it might inactivate PAF and oxidized PL to minimize the pathology of these lipids in sepsis. PAF-AH has a proatherogenic role due to its ability to generate PLC and oxidize FA that recruit and activate leukocytes and induce apoptosis. Thus, pharmacological inhibition of PAF-AH may be of importance in the prevention of atherosclerosis. The intracellular PAF-AH II (PLA_2V11B) hydrolyzes sn-2 acyl-chains of up to five carbons and facilitates transfer of acetyl group of PAF to lysoPAF and ceramide in a CoA-independent manner through its transacylase activity. PAF-AHII is highly expressed in liver and kidney where it plays a pivotal role in defense against oxidative stress by degradation of oxidized PLs in membrane. PAF-AHII-null mice are extremely sensitive to chemicals that induce oxidative stress and show elevated levels of esterified 8-iso-$PGF_2\alpha$ in the liver. Furthermore, transgenic mice overexpressing PAF-AHII are protected from ischemic injury (Umemura et al., 2007).

There are two distinct members of the lysosome PLA_2 family, aiPLA2 (peroxiredoxin6) and macrophage lysosomal PLA_2 ($LPLA_2$), which are highly homologous to lecithin:cholesterol acyltransferase. Lysosome aiPLA$_2$-null mice are sensitive to oxidative stress, most likely due to the absence of the enzyme's glutathione peroxidase activity. $LPLA_2$-null mice show a marked accumulation of PL in macrophages with features of foam cells and lamellar inclusion, which is a hallmark of phospholipidosis. Recently, a new adipose-specific PLA_2 ($AdPLA_2$) expressed in WAT has been identified. This enzyme exhibits both sn-1 and sn-2 phospholipase activities and releases FA from WAT TG stores (Jaworski et al., 2009). Adipose specific $AdPLA_2$-null mice have reduced WAT and TAG contents but normal adipogenesis with increased FA β-oxidation within adipocytes (Jaworski et al., 2009). $AdPLA_2$-null mice have a higher rate of lipolysis due to a decrease in

adipose PGE_2 level, which activates the EP3-coupled Gαi receptor, which counteracts cAMP-stimulated lipolysis. Thus $AdPLA_2$ plays an important role in supplying AA for PGE_2 synthesis in WAT. Congenic $AdPL_2$-null/ob/ob mice are hyperphagic, yet lean, with increased energy expenditure, but have ectopic TAG storage and insulin resistance reminiscent of human type II diabetes. It is believed that $AdPLA_2$ has a dual role in WAT adipogenesis through both supplying AA for PGE_2 synthesis and recruitment of M1 macrophages that induce the cytokine-chemokine cascade during inflammation.

It is very apparent that the once-thought only role of PLA_2 enzymes solely in the initiation of inflammation needs to be modified because the results of recent studies that implicate the 30 PLA_2 family members as important enzymes in supplying bioactive lipids that control cellular lipid metabolism. However, the cytosolic $cPLA_2$ is the key regulator of AA-mediated eicosanoid metabolism; both the independent $iPLA_2$ and secreted $sPLA_2$ also function in the inflammatory process. Many of the PLA_2 enzymes exhibit transacylase activity in addition to phospholipase and lysophospholipase activities. Their broad roles in eicosanoid and intermediary metabolism are evident from their diverse roles in FA metabolism. Phospholipase A_2 enzymes has an important role in membrane PL remodeling, selective regulation of FA transport proteins (e.g. FABP, ACBP, FATP and ASCL) channeling to meet the metabolic needs of cells, and the systemic energy needs of the organism. Alteration of these processes can lead to diseases associated with MetS. The understanding of the functional role of PLA_2 in metabolism in the liver is in its infancy. To further identify PLA_2-generated lipid metabolites and especially eicosanoids' role in metabolic disease will require a comprehensive proteomic, lipidomic and genomic approach to provide a metabolomic picture of eicosanoids and their pathways in control of cellular metabolism. These methods will be of an immense value in the future in our dissection of eicosanoids' role in metabolism and their permutation in MetS (Sabido et al., 2012).

3. METABOLISM OF EICOSANOIDS IN MetS

MetS is a cluster of metabolic and physiological abnormalities that increases an individual's risk for CVD, type II diabetes, obesity, and NAFLD, which includes symptoms of hyperglycemia, insulin resistance, hypertension, hypertriglyceridemia, hyperlipidemia, and hypercholesterolemia. The causes of these abnormalities are currently believed to be dysfunction in

lipid metabolism and persistent subacute inflammation caused by alterations in lipid signaling networks that link the immune system and metabolism in metabolic diseases. Although cytokines and chemokines play significant role in the abnormalities of MetS, bioactive lipids may be the early link between inflammation and MetS since drugs that target the synthesis of eicosanoids in inflammation have efficacy in the treatment of MetS. Of equal importance is the observation that dietary ingestion of ω3-PUFAs that produce less-potent eicosanoids and beneficial resolvins reduces the severity of inflammation and many symptoms of MetS. Thus, eicosanoids may provide a common link between inflammation and MetS and targeting selective eicosanoid pathways may provide unexplored opportunities in the current treatment of not only CVD but also other diseases of MetS and NAFLD.

Release of AA from PLs (PI, PC, PE, PS, or CL) by the action of specific and selective PLA_2 isoforms can be metabolized by cyclooxygenase, LOX, and cytochrome P450 pathways to produce potent eicosanoids and lipid autacoids. These pathways produce a variety of bioactive eicosanoids including PGs, thromboxanes (TXs), LTs, LX, epoxyeicosatetraenoic acid (EET), hydroxyeicosatetraenoic acid (HETE) and isoprostanes (Fig. 5.2). These eicosanoids elicit their paracrine, autocrine, or intracrine effects through either specific GPCRs, activation of NHRs transcription factors, or alteration of specific eicosanoid enzymes directly.

3.1. Synthesis of Prostaglandins in Intermediary Metabolism

PG and TXs are synthesized from two different PG endoperoxide synthase, prostaglandin endoperoxide H synthase (PGHS)-1 and PGHS2 that catalyze two distinct reactions, a cyclooxygenase and a peroxidase reactions. COX bisoxygenates arachidonic acid, leading to two molecules of oxygen being inserted into AA to yield protaglandin peroxidase. The peroxidase activities of PGHS is performed by a glutathione peroxidase producing PGHS that is rapidly converted by specific synthase to produce PGD_2, PGE_2, $PGF_2\alpha$, prostacyclin (PGI_2), or TXA_2. There are two different PGHS (PGHS1 and PGHS2) with different cyclooxygenase activities (COX1, COX2) and different substrate specificities and patterns of regulation. High concentration of palmitic acid in obesity and T2DM stimulates the inflammatory PGHS2 and inhibits the constitutive PGHS1. Cyclooxygenase activity is by two COX monomers, one an allosteric activator site that binds heme and the other a catalytic site. COX activity is regulated by different FAs that elicit a stimulatory or inhibitory

effect on AA oxygenation, depending on the FA and PGHS isoforms. Many COX inhibitors that include nonsteroidal anti-inflammatory drugs (NSAID) inhibit both COX1 and COX2, while Coxibs drugs show selectivity in inhibition of COX2. Many of the COX inhibitors are metabolized by cytochrome P450s CYP2C subfamily members. It is also of interest that antiinflammatory ω3-PUFAs are poor substrates for COX isoforms with EPA not being metabolized by COX1 and only weakly metabolized by COX2 (Smith et al., 2011). Thus the differential regulation of COX1 and COX2 activities by saturated SFAs and uSFAs can lead to production of pro- or antiinflammatory eicosanoids. Biologically active 2-series prostanoids are produced in the presence of palmitic acid by COX2, while antiinflammatory eicosanoids would be produced by COX2 or diverted to LOX pathway to produce less-potent LTs. Thus the high consumption of ω-6 linoleic acid seen in a Western diet would produce pro-inflammatory prostanoids and endocannabinoids such as anandamide and 2-acylglycerol.

It is rather surprising that the metabolic phenotypes of COX1 and COX2-null mice have not been completely characterized thus leaving a void in our understanding of prostanoids in the control of intermediary metabolism in MetS. This is especially surprising considering that PGE_2 has a pivotal role in adipocyte differentiation and adipogenesis. Of equal importance is the different roles of COX1 and COX2 in the production of different prostanoids, with COX1 coupled with synthesis of TXA_2, $PGF_2\alpha$ and PGE_2 production, and COX2 preferentially channeling to PGI_2 and PGE_2 synthesis (Smith et al., 2011). This selective channeling is evident in inflammation since COX1- and COX2-null mice have different responses in inflammation with COX1-null showing an attenuated response to AA-induced ear edema, while COX2-null mice having a similar response as wild-type mice. COX2 has a major role in acute and chronic inflammation as well as in the resolution phase of inflammation where antiinflammatory PGD_2 and 15-deoxyPGJ_2 levels increase, while proinflammatory PGE_2 levels drop. The role of COX2 in atherosclerosis is uncertain since COX1-null mice have a marked reduction of lesion development in the apoE lipoprotein (APOE)-null mice (McClelland et al., 2009), while COX2 PGI_2 in PGI_2 receptor knockout mice show early development of atherosclerosis in hyperlipidemic mice.

The different profile of prostanoids produced by COX1 and COX2 is largely determined by which COX isozyme is expressed in the cells and under what condition. This is clearly evident in normal macrophages that

produce equal amounts TXA_2 and PGE_2, but upon activation, the level of PGE_2 dramatically increases. PGE_2 is the most abundant prostanoid synthesized from PGH_2 by cytosolic PGE-1 synthase (PGES1) and microsomal PGES1 (mPEGS-1) or PGES2 that requires reduced glutathione (GSH) for its activity. Like COX2, microsomal PEGS is induced by cytokines and growth factors and is inhibited by glucocorticoids. PGE_2 is involved in three of five cardinal signs of inflammation: rubor (redness), tumor (swelling), dolar (pain), calor (heat), and function laesa (loss of function). In inflammatory model of angiogenesis, mPGES-1-null mice show reduced vascular endothelial growth factor (VEGF), suggesting that PGE_2 and VEGF cooperate in angiogenesis. PGE_2 is actively transported from cell by multidrug resistance protein 4 (MRP4), and binds locally to one of four cognate receptors (EP1-EP4) (Jania et al., 2009). Both EP3 and EP4 are widely expressed in most tissues, therefore the interpretation of EP4-null mice phenotype are difficult to reconcile without the generation of tissue-specific knockout mice.

PGI_2 is the most important cardiovascular prostanoid produced by vascular endothelial and vascular smooth muscle (VSM) cells. Prostacyclin synthase is a member of the cytochrome P450 family (CYP8A1), which colocalizes with COX1, yet COX2 is the predominate source of PGI_2. Endothelial PGI_2 relaxes VSMC and inhibits platelet aggregation, before it is inactivated to 6-keto $PGF_2\alpha$. PGI_2 possibly mediates some of its effects through activation of PPARβ, similar to PPARγ activation by 15-deoxyPGD_2. PGI_2 receptor IP-null mice show accelerated atherogenesis with increased platelet activation and enhanced leukocyte attachment to the vessel in ApoE-null mice (Kobayashi et al., 2004). T cells in adipose tissue are believed to have a significant role in obesity-induced inflammation by modifying adipose tissue macrophage (ATM) numbers and macrophage phenotype to M1 macrophages that secrete pro-inflammatory tumor necrosis factor (TNF)-α and IL1. CD4+ Th1 cells produce inflammatory cytokines, the Th2 cells produce anti-inflammatory cytokines, and regulatory T-cells secrete anti-inflammatory signals that inhibit macrophage migration and induce M2-like macrophage differentiation. Thus PGE_2 promotes macrophage differentiation of monocytes to the M1 macrophages and Th1 T cell phenotype, while PGI_2 promotes production of M2 antiinflammatory macrophages and Th2 cells.

PGD_2 is the major prostanoid synthesized in peripheral tissues by the cytosolic lipocalin-type PGD synthase (L-PGDS) found in mast cells, leukocytes and Th2 cells. PGD_2 is further metabolized to $PGF_2\alpha$ and a series of cyclopentanone PGs, $PGJ_2\Delta_{12}$, and 15-PGJ_2. PGD_2 is the predominate

prostanoid in activated mast cells and appears to mediate its proinflammatory effects through DP1 and DP2 GPCRs. PGD synthase is a member of the commonly known drug metabolizing enzyme glutathione-S-transferase family (α, μ, and π classes) that have the ability to convert PGH_2 to a mixture PGD_2, PGE_2, and $PGF_2\alpha$ in the presence of GSH. The PG synthase enzyme glutathione-S-transferase activity most likely complements the glutathione peroxidase activity of COX enzymes, suggesting a close relationship between drug and eicosanoid metabolism. This suggests a potential source of adverse drug toxicity when glutathione levels are depleted in oxidative stress, which can lead to reduced export of toxic drugs and inhibition of prostanoid synthesis.

PGF synthase produces $PGF_2\alpha$ that activates $PGF_2\alpha$ FP receptor GPCR coupled to Gα(q/11), leading to elevation of intracellular calcium mobilization. $PGF_2\alpha$ can be metabolized to the major plasma metabolite 15-keto dihydro$PGF_2\alpha$ by members of the aldol-reductase family. The aldo-keto reductase (AKR) families are widely distributed, consisting of 15 families that metabolize aldehydes, steroids, monosaccharides, aromatic hydrocarbons and prostanoids in the presence of NADPH. A recent study identified that AKR1B7 has a significant role in the detoxification of lipid peroxidation malondialdehyde (MDA), which functions as a chemotactic agent in attracting macrophages and neutrophils to sites of injury (Ge et al., 2011). Surprisingly, adenovirus overexpression of AKR1B7 in liver of diabetic db/db mice lowered blood glucose, hepatic gluconeogenesis, hepatic TAG, and cholesterol. Thus AKRs have dual roles in prostanoid metabolism and intermediary lipid and carbohydrate metabolism. In addition, functional coupling of COX2 and AKR1B7 to produce $PGF_2\alpha$ has been demonstrated in HEK 293 cells. Thromoboxane B_2 (TXB_2) is synthesized in platelets by COX1 as TXA_2, which is nonenzymatically degraded to TXB_2 with the parallel production of MDA and 12-hydroxyeicosatrienoic acid (12-HHT). TXA_2 signal through the thromboxane TP receptor coupled to Gq, $G_{12/13}$, and other G proteins to influence Rho GEF, adenylate cyclase and PLC that mediate platelet aggregation, adhesion, VSMC contraction, and proliferation during inflammation. Several other eicosanoids including isoprostanes, produced by oxidation of prostanoids and HETEs are potent agonists of TP, while EETs produced by cytochrome P450 epoxygenase are potent antagonists (Behm et al., 2009). TXs are synthesized by TX synthase, a member of the cytochrome P450 family identified as CYP5.

It is apparent that prostanoids can either promote or attenuate acute inflammation through channeling of PGH2 from either COX1 or COX2 to different synthase. Therefore, it is unknown how and why some drugs, that

target the prostanoid pathway, are so effective in the treatment of inflammation, CVD, colorectal cancer, asthma, arthritis and thrombosis, but ineffective against the progression of these diseases. Inhibition of one key downstream pathway would have two benefits with regard to drug development in the treatment of inflammatory and metabolic disease. Blockage of the synthesis of proinflammatory prostanoids would inhibit the amplification of the lipid autacoid-cytokine-chemokine cascade and divert the prostanoid substrates through other pathways and thus reduce the probability of toxicity. This tenet is seen in aspirin inhibition of platelet COX1-derived TXA_2 that amplifies platelet aggregation, while aspirin inhibits amplification and persistent activation of platelet aggregation. Targeting the eicosanoid pathway in the treatment of metabolic disease may offer similar benefits of reduced toxicity and attenuation of sustained activation and amplification of disease pathways. Presently, we do not completely understand the role of prostanoids synthesis in the progression of obesity, fatty liver, and diabetes, unlike our detailed understanding of eicosanoid metabolism, in the inflammatory etiology of CVD disease. With regard to prostanoid metabolism, few studies have explored the role of these pathways in obesity and fatty liver disease, which is rather perplexing given the rapid induction of COX2 by peroxides, oxidant stress, NF-κβ cytokines (TNFα, transforming growth factor (TGF)-β1) and chemokines (IL1β, IL6) through activation of lipid autacoid-cytokine-chemokine cascade. COX2 inhibitors Celecoxib and NA-398 inhibited the progression of NAFLD. (Yu et al., 2006). Furthermore, a recent study has provided evidence that drugs targetting eicosanoid metabolism may be effective in the treatment of MetS. Aspirin that targets COX enzymes also has the ability to activate AMPK, the pivotal enzyme in the integration of carbohydrate and lipid metabolism (Hawley et al., 2012). Activation of AMPK inhibits ACC, the rate-limiting enzyme in lipogenesis, and activates FA oxidation. Similarly, niacin, a precursor to NADH and NADPH used in the treatment of MetS dyslipidemia induces COX1-dependent PGD_2 and PGE_2 and COX2-dependent PGE_2 production, which causes vasodilation. Thus there is a grave need to explore the prostanoid biosynthetic pathway in obesity, fatty liver, and diabetes with regard to alterations in intermediary metabolism in MetS.

3.1.1. Leukotrienes Synthesis in Intermediary Metabolism

LTs, LX, hydroperoxyeicosatetraenoic acid (HPETE), and eoxins are synthesized by 5-, 12-, and 15-LOX that lipoxygenate AA by adding molecular oxygen to form 5-, 12-, or 15-HPETEs, respectively. 5-HPETE is further

metabolized by 5-LOX to LTA_4, which can be metabolized to LTB_4 or the glutathione containing slow-reacting substances of anaphylaxis (LTC_4, LTD_4, LTE_4). 12-LOX produces 12-HETE and 15-LOX produces 15-HETE. LXA_4 and LXB_4 are produced through transcellular metabolism and have antiinflammatory properties during the resolution of inflammation.

5-LOX is primarily expressed in hematopoietic cells, such as leukocytes, mast cells, dendritic cells, and lymphocytes and its expression is induced by growth factors and cytokines (Haeggstrom & Funk, 2011). 5-LOX activity requires Ca^{2+} and PC and its activity is induced by FA hydroperoxides that are controlled by glutathione peroxide, thus linking LT synthesis and prostanoid synthesis. 5-LOX activity is regulated by serine phosphorylation by MAPK2, ERK2, and protein kinase A (PKA). Both MAPK2 and ERK2 phosphorylation of 5-LOX induces its translocation to the nucleus, while PKA-mediated phosphorylation inhibits its translocation. For optimal activity, 5-LOX binds 5-lipoxygenase activating protein (FLAP), which stimulates AA utilization and activity to produce LTA_4. LTA_4 hydrolase is widely expressed in most tissues unlike 5-LOX, which is restricted to hematopoietic cells. LTA_4 hydrolase is a bifunctional enzyme having epoxide hydrolase activity and aminopeptidase activity. LTA_4 inhibits LTA_4 hydrolase activity, but not its aminopeptidase activity. During tissue damage, collagen of the extracellular matrix is broken down to produce the highly chemotactic tripeptide (proline-glycine-proline), which is inactivated by the aminopeptidase activity of LTA_4 hydrolase. (Haeggstrom & Funk, 2011). Thus LTA_4 inhibits the synthesis of pro-inflammatory LTB_4 by blocking LTA_4 hydrolase activity without inhibiting the aminopeptidase activity of LTA_4 hydrolase.

Presently, we do not know the importance of eicosanoid metabolism and the role of prostanoids, LTs and cytochrome P450 (CYP) AA metabolites in the initiation and progression of MetS and their importance in NAFLD, obesity and insulin resistance. However, several recent studies pointed toward the role of LOX pathways in fatty liver disease based on: (1) the aberrant expression of Alox5 gene in liver during the progression of chronic liver disease (Titos et al., 2010), (2) the protection against hepatic steatosis by administration of 5-LOX inhibitors (Lopez-Parra et al., 2008), and (3) protection against liver inflammation and fibrosis by coadministration of 5-LOX and COX2 inhibitors (Horrillo et al., 2007). In addition, several recent studies have used LOX knockout mice to explore the role of LT metabolites in the control of lipid metabolism and inflammation in liver disease.

The Alox5 gene is highly expressed in several models of liver diseases (El-Swefy & Hassanen, 2009; Lopez-Parra et al., 2008; Titos et al., 2010).

Alox5-null mice show a lower degree of hepatic steatosis that a differential regulation of lipid metabolism genes, including the lipogenic factors, Lipin1, c/EBP, Fasn, Acly, and Elovl6 (Titos et al., 2010). The mechanisms by which 5-LOX metabolites initiate liver dysfunction and damage have not been completely identified. It is known that 5-LOX metabolites induce NF-κβ, which increases the expression of proinflammatory cytokines and chemokines (MCP-1, IL1, IL6, and IL8) and cell survival (TNFα). The prevention of steatosis in Alox5-null mice is due to downregulation of FA synthase and ATP-citrate lyase as well as other lipogenic genes and inhibition of microsomal triglyceride transfer protein (MTP), VLDL-TG, and apolipoprotein B secretion (Horrillo et al., 2010). Thus in hepatic steatosis and steatohepatitis, 5-LOX increases lipogenesis and inhibits TAG transport leading to accumulation of TAGs in the liver (Martinez-Clemente et al., 2010). Although 5-LOX-generated LTB_4 has a pivotal role in inflammatory chemotaxis and the progression of steatosis to steatohepatitis, it is unknown whether LTB_4 has a role in the initiation of hepatic steatosis. LTB_4 receptor1 (BLT1)-null mice are resistant to HF-diet-induced obesity and insulin resistance. This is due to reduced accumulation of M1 ATMs and increase in adipose M2 ATMs that prevent lipolysis but promote hepatic triglyceride accumulation and liver insulin resistance (Spite et al., 2011). It is unknown whether BLT2 highly expressed in the liver also has a role in hepatic steatosis.

Although 5-LOX LTB_4 appears to be the most relevant metabolite in hepatic inflammation, the cysteine LTs have a significant role in inflammation and hepatocyte survival (LTC_4, LTE_4) and stellate cell activation (LTD_4) (Martinez-Clemente, Ferre, Gonzalez-Periz, et al., 2010; Titos et al., 2010). LTC_4 synthase is the committing step in cys-LT synthesis through the conjugation of LTA_4 with glutathione. LTC_4 synthase is a member of the microsomal GSH transferase (MGST) family of proteins that have LTC_4 synthase activity and peroxidase activity toward hydroperoxides (Haeggstrom & Funk, 2011). LTC_4 is sequentially metabolized by γ-glutamyl transpeptidase to produce LTD_4 (cys–gly), and peptidase to produce LTE_4. The cysteinyl LTs increase vascular permeability and plasma leakage of vessels leading to edema. There are no studies on the role cysLT has in either MetS or NAFLD. Even though LTA_4 hydrolase-null mice have been generated, but the role of LTA_4 deficiency in fatty liver, obesity, or metabolic disease has not been reported.

There are two forms of 12-LOX, platelet and leukocyte forms in mice. In humans, the 12-LOX and human reticulocyte 15-LOX isoform produce similar products and are often referred to as 12/15-LOX. Because of species

difference in expression, such as mice expressing the 12-LOX but not 15-LOX, it is difficult to interpret the results of animal studies to humans. The human 12/15-LOX produces a series of important lipid mediators, including 12-HPETE and 15-HPETE, that are further metabolized to 12-HETE and 15-HETE, respectively (Dobrian et al., 2010). The 12/15-LOX also oxygenates linoleic acid (C18:2n6) to produce 9-hydroxyoctadecadienoic acid (HODE) and 13-HODE, as well as hydroxylating FAs esterified in PLs. Also, 9-HODE and 13-HODE are able to activate the toll-like receptor (TLR)-4 in macrophages, and 12/15-LOX metabolites induce production of pro-inflammatory cytokines and chemokines, such as MCP-1, IL6, IL8 and TNFα. The 12/15-LOX-generated hydroperoxides can also serve as precursors in the formation of secondary lipid mediators called LX, hepoxilins (HX), and trioxlins, and antiinflammatory lipid mediators from ω3-PUFA known as resolvins and protectins (Spite et al., 2011) (Fig. 5.3).

HX are formed from 12-LOX, 12-HPETE through an intramolecular rearrangement of –OOH group to form a hydroxyl group at C8 (HXA3), C10 (HXB3) by a putative isomerase or an epoxide at the 11,12 position by epoxide hydrolase to form three-hydroxyl-group-inactive metabolites called trioxilins. HX are early signals of inflammation while the 15-LOX-pathway-produced LX (LXA_4 and LXB_4) have both anti-inflammatory resolving abilities (Spite et al., 2011). The formation of LX begins with 15-HETE that is converted to LXA_4 by 5-LOX through transcellular biosynthesis. It is significant that aspirin acetylation of COX2 inhibits COX2 from producing PGH2, but COX2 retains oxygenase activity to produce 15(S) HETE that can be converted to antiinflammatory LX. Over expression of 12/15-LOX in the endothelium increases atherosclerotic plaques, while overexpression in macrophages protects against atherosclerosis in mice. Further perpetuating atherogenesis is angiotensin II that upregulates 12/15-LOX expression in macrophages and endothelium leading to vasoconstriction. It has been reported that polymorphism in the Alox12 gene is associated with subclinical atherosclerosis and serves as a biomarker of disease in families with type II diabetes mellitus (T2DM) (Haeggstrom & Funk, 2011).

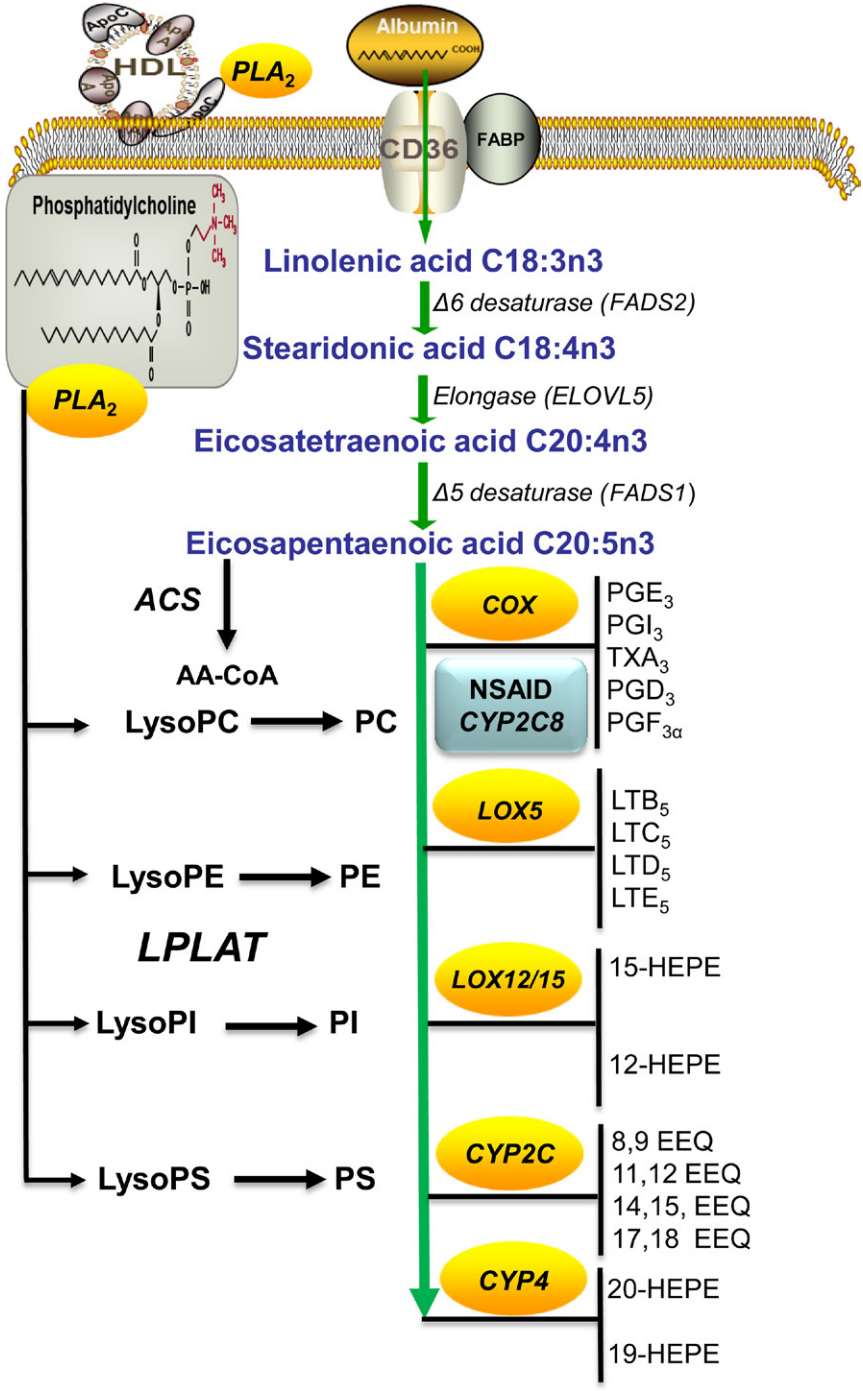
Normal blood glucose levels are maintained largely by β-cell insulin and α-cell glucagon secreted from the pancreas. Insulin promotes glucose uptake by adipose, liver and muscle tissue and promotes glycolysis, glycogen synthesis and inhibition of hepatic gluconeogenesis. Both type I and type II diabetes are associated with a significant loss of β-cell function and thus insulin production. Inflammation has a pivotal role in β-cell dysfunction, and upregulation of 12/15-LOX by hyperglycermia and inflammatory

cytokines have a central role in diabetes and obesity. Furthermore, both 5-LOX and 12/15-LOX have been implicated in MetS and NAFLD in humans and a recent plasma lipidomic signature of NASH revealed a stepwise increase in 5-LOX 5-HETE, 8-HETE, and 12/15-LOX 15-HETE in the progression of NAFD to NASH (Puri et al., 2009), further supporting the role of LOX in insulin resistance, obesity and fatty liver disease.

3.1.2. Eicosanoids of the P450 Epoxygenase and Omega Hydroxylase Pathways

The third pathway for the metabolism of eicosanoid is mediated by cytochrome P450s of the epoxygenase pathway (CYP2C) and FA omega hydroxylase (CYP4). These drug-metabolizing pathways were once thought to have little function in the control of metabolism; however, recent studies have shown their importance in the disease pathology of MetS. In addition, many of the commonly prescribed antiinflammatory drugs that target PG and LT eicosanoid pathways are metabolized by members of the drug-metabolizing cytochrome P450 family (Figs 5.2 and 5.4). It is also apparent that there are stark similarities in the drug-metabolizing enzymes of phase I oxidation, phase II conjugation, and phase II transport with enzymes of eicosanoid metabolism, which establishes a link between eicosanoid and drug metabolism in metabolic diseases.

Unlike COX and LOX that insert molecular oxygen into AA and therefore function as dioxygenase, CYP enzymes are monoxygenase inserting one oxygen atom into the substrate and the other in the formation of water. CYPs are able to efficiently use AA and ω3-PUFAs as substrates similar to LOX; however, because COX enzyme metabolizes ω3-PUFAs less efficiently, it is likely that LA-derived EPA (C20:5n3) is preferentially metabolized by CYP or LOX pathways. In addition, neither linoleic acid nor LA is used by either the LOX or COX pathways, but are efficiently metabolized by the CYP pathway. CYP enzymes catalyze the hydroxylation, epoxidation and allylic oxidation of FAs (Oliw et al., 1996). CYP2 members metabolize AA to four regioisomeric cis-epoxyeicostetraenoic acids (5, 6-; 8, 9-; 11, 12-; and 14, 15-EET), with each of these regioisomers forming R, S or S, R enantiomers. This regioselectivity and stereoselectivity of EETs is CYP isoform specific. Human CYP2C8 exclusively metabolizes AA to 14,15- and 11,12-EETs in a ratio of 1.3:1, while CYP2C9 shows less region- and stereoselectivity. CYP2J epoxygenase is highly expressed in the heart and produces all four regioisomers as a mixture of racemers. The human CYP2S1 P450 produces 12-HHET, MDA, 13-HODE, 5-oxo-EET,


HDL
PLA2
Albumin
COOH
CD36
FABP
Phosphatidylcholine
Linolenic acid C18:3n3
Δ6 desaturase (FADS2)
Stearidonic acid C18:4n3
Elongase (ELOVL5)
Eicosatetraenoic acid C20:4n3
Δ5 desaturase (FADS1)
Eicosapentaenoic acid C20:5n3
PLA2
ACS
AA-CoA
LysoPC
PC
LysoPE
PE
LPLAT
LysoPI
PI
LysoPS
PS
COX
PGE3
PGI3
TXA3
NSAID
CYP2C8
PGD3
PGF3α
LOX5
LTB5
LTC5
LTD5
LTE5
LOX12/15
15-HEPE
12-HEPE
CYP2C
8,9 EEQ
11,12 EEQ
14,15, EEQ
17,18 EEQ
CYP4
20-HEPE
19-HEPE

and 12-oxo-EET (Bui et al., 2011). Allylic oxidation of AA by other P450 isoforms produces hydroxylated metabolites containing cis- and trans-conjugated dienol (5, 8-; 9-; 11-; 12- and 15-HETE). The 12- and 15-HETE are similar to the 12/15-LOX AA metabolites and therefore are believed to have similar function in the liver, adipose tissue and pancreas. These allylic oxidation products are produced by several CYP isoforms including CYP1A2, CYP3A4, CYP2C8 and CYP2C9.

The human epoxygenase CYP2C8, CYP2C9 and CYP2J2 as well as soluble epoxide hydrolase (sEH) that converts EET to dihydroxyeicosatrienoic acid (DHET) are highly polymorphic and several variants have been associated with individual risk for stroke, hypertension, atherosclerosis, myocardial infarction, and cancer (Zordoky & El-Kadi, 2010). EETs are also known as endothelial hyperpolarizing factor and their synthesis is initiated in the vascular bed in response to bradykinin and acetylcholine activation of BK channels in VSMC leading to hyperpolarizations and VSMC relaxation. EETs activate endothelial nitric oxide synthase (eNOS) to cause vasodilation. In the heart, EETs regulate L-type Ca^{2+} ATP-sensitive potassium (KATP) and Na^{+} channels thus improving functional recovery from ischemia/reperfusion (I/R) injury as shown by heart-specific overexpression of CYP2J2.

The ω-3 PUFAs, EPA and DHA are converted by epoxidation and hydroxylation to both 5-regioisomeric epoxyeicosatetraenoic acid (EEQ) and ω-HEPE (Fig. 5.3) (Westphal et al., 2011). The beneficial effects of dietary consumption of ω3-PUFAs are through downregulation of the inflammatory response, and well-established attenuation of lipogenesis. This

Figure 5.3 ***Metabolism of linolenic acid to anti-inflammatory eicosanoids.*** The synthesis of anti-inflammatory eicosanoids from ω-3 polyunsaturated fatty acids (PUFA) begins with the desaturation and elongation of the ω-2 linolenic acid to produce eicosapentaenoic acid (EPA). Less potent series-3 prostanoids and series-5 leukotrienes are produced from EPA. However, the CYP2 epoxygenase produces a series of epoxyeicosatetraenoic acids (EEQ) at a 10-fold higher rate than the metabolism of AA, while CYP4 ω-hydroxylase produces hydroxyeicosapentaenoic acid (20-HEPE) at an approximate twofold higher rate than AA and increased rate of ω-1 over ω-hydroxylation of EPA compared with AA. In vivo, the EEQ surpasses the role of EETs as endothelium-derived hyperpolarizing factors; however, the role of CYP4-derived 19-HEPE over the 20-HEPE in the vasoconstriction and proinflammatory role of AA are presently uncertain. It will be of particular interest to determine the beneficial effects of EPA metabolites in metabolic disease considering that PUFAs suppress lipogenesis and EPA alleviates obesity-induced insulin resistance by upregulation of glucose transporters GLUT2, GLUT4, insulin receptor substrates IRS-1 and IRS-2 and reduction in cholesterol ester transfer protein (CETP). (For color version of this fi gure, the reader is referred to the online version of this book).

is the reason we explore how their metabolisms influence metabolic processes of MetS. Both EPA and DHA are efficiently metabolized by CYP-dependent epoxidation and hydroxylation to both Omega and epoxide metabolites of EPA and DHA (Konkel & Schunck, 2011). Several members of the CYP2 family (CYP2C8, C9, C18, C19 and CYP2J2) metabolize EPA (C20:5n3) to 5-regioisomeric EEQ and DHA (C22:6n3) to 6-regioisomeric epoxydocosapentaenoic acid with equal or higher catalytic activities, but different regioselectivity for EPA versus AA (Fer et al., 2008). CYP2C8 metabolizes AA to 11, 12- and 14, 15-EET, while EPA is metabolized to 17, 18-EEQ and DHA to 19,20-EDP. The heart-specific CYP2J2 metabolizes EPA and DHA at rates 10- and 2-fold greater, respectively, than its rate of AA epoxidation. Presently, the biological activities of EPA and DHA epoxides are similar but have more powerful effects than AA-formed EETs since BK channel activation by 17,18-EEQ greatly exceeds that of 11,12-EET, the strongest AA metabolite activator of BK channels (Westphal et al., 2011). Also, DHA-derived epoxides are 1000-fold more potent than EET in activating BK channels in rat coronary arterioles.

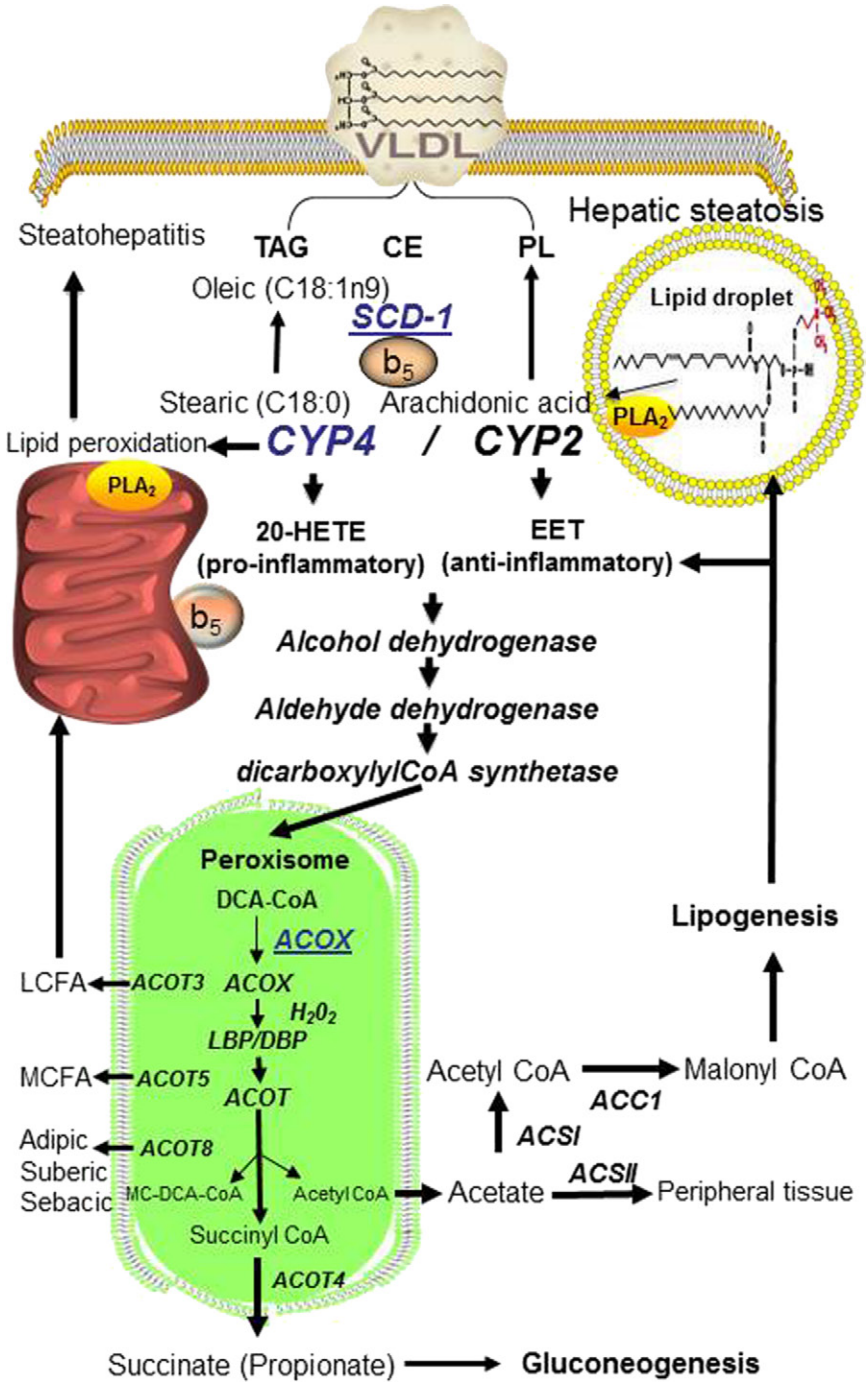
The other CYP eicosanoid metabolizing P450s are members of the FA ω-hydroxylase family (CYP4), which consists of multiple human subfamilies (CYP4A, CYP4B, CYP4F, CYP4Z, CYP4X, and CYP4V), but only members of CYP4A and CYP4F have been studied in detail in regard to eicosanoid metabolism. The CYP4 isoforms ω-hydroxylate the subterminal carbon of AA to produce 20-HETE and ω-hydroxylated LCFAs and eicosanoids that are metabolized by peroxisomal β-oxidation (Fig. 5.4). Hydroxylation of AA is performed by other CYP enzymes forming a series of subterminal regioisomeric HETE, 16-,17-, 18-, and 19-HETE is catalyzed by the ethanol-inducible CYP2E1 (18-, 19-HETE), CYP1A1 and CYP1A2 (16-,17-, 18-HETE); CYP2J9 that produces exclusively 19-HETE; and CYP4F22 (18-HETE) (Nilsson, Ivanov, & Oliw, 2010). A recent study on the activity of the 19-hydroxy-PGH2 CYP4F8 and CYP4F22 P450 in the metabolism of AA and ω3 PUFA showed that CYP4F22 produces 18-HETE, and CYP4F8 metabolizes ω3-PUFA to 8,9- and 11,12-epoxyalcohols (HEETS-hydroxyepoxyeicosatrienoic acid) (Nilsson et al., 2010).

The human CYP4A11 ω-hydroxylase produces 20- and 19-HETEs in a ratio of 90:10, while members of the human CYP4F subfamily CYP4F2, CYP4F3A, CYP4F3B show strict regioselectivity in the ω-hydroxylation of AA (Konkel & Schunck, 2011). The functional role of other CYP4 subfamily members (CYP4F11, CYP4F12, CYP4V2, CYP4Z1, and CYP4F22) in the metabolism of AA and ω-PUFA has not been extensivly

studied. ω-hydroxylate AA (20-HETE) has been shown to have a vital role in hypertension through its ability to cause vasoconstriction by inhibiting the K^+ BK channel, stimulation of Rho kinase, and activation of L-type calcium channels. Vascular production of 20-HETE induces endothelial dysfunction and hypertension through reduced endothelial nitric acid oxidase (eNOS) activity and activation of NF-κβ (Wu, Cheng, et al., 2011). Endothelial dysfunction has been shown to be correlated with the level of urinary 20-HETE. Genetic polymorphisms in CYP4A11 and CYP4F genes are associated with hypertension, stroke and coronary endothelial dysfunction (Fava et al., 2012; Stec et al., 2007; Zordoky & El-Kadi, 2010). CYP4A11, CYP4F2, CYP4F3a, and CYP4F3b efficiently metabolize EPA and DHA (Fer et al., 2008) with CYP4A11 showing a dramatic shift in ω/ω-1 hydroxylase activity ratio of 4:1 for AA to 1:3 with EPA and 1:2 with DHA. This is of particular interest since 19-HETE inhibits 20-HETE-mediated vasoconstriction and endothelial dysfunction, and thus ω3-PUFA may shift CYP4A production of proinflammatory 20-HETE to beneficial effect of 19-hydroxylated EPA or DHA.

CYP4F2 not only produces 20-HETE from AA but also hydroxylates DHA at twofold higher rate than either AA or EPA, while CYP4F3A and CYP4F3b ω-hydroxylate AA and DHA at a similar rate with less activity toward EPA (Fer et al., 2008). Both CYP4F8 and CYP4F12 metabolize EPA and DHA by epoxidation of the ω-double bond to produce 17,18-EEQ and 19,20-EDP, respectively (Stark, Dostalek, & Guengerich, 2008). The brain- and thymus-specific CYP2U1 ω-hydroxylase efficiently ω-hydroxylates ALA, AA, EPA, and DHA (Konkel & Schunck, 2011). Unlike the epoxidation of EPA and DHA that seem to have similar activities as AA-derived EET, the function of ω-hydroxylated metabolites of EPA and DHA are not known, therefore it will be of importance to determine if ω-hydroxylated EPA and DHA have reduced vasoconstrictive and proliferation abilities.

Besides the use of several common enzymes of drug metabolism in eicosanoid metabolism as exemplified by CYP2C epoxygenase and CYP4 ω-hydroxylase, the channeling and partitioning of AA through these three pathways can have a significant role in inflammation, drug metabolism, and intermediary metabolism. This is evident by the partitioning and channeling of EPA that is not efficiently metabolized by COX, but efficiently metabolized by LOX, CYP2 epoxygenase, and CYP4 ω-hydroxylase. Of equal importance is that drugs used to control eicosanoid metabolism (naproxen, ibuprofen, indomethacin, rofecoxib and diclofenac) are metabolized by CYP2 members of the epoxygenase pathways (Fig. 5.2), suggesting


VLDL
Steatohepatitis
TAG
CE
PL
Hepatic steatosis
Oleic (C18:1n9)
SCD-1
b5
Lipid droplet
Stearic (C18:0)
Arachidonic acid
PLA2
Lipid peroxidation
CYP4 / CYP2
PLA2
20-HETE
(pro-inflammatory)
EET
(anti-inflammatory)
b5
Alcohol dehydrogenase
Aldehyde dehydrogenase
dicarboxylylCoA synthetase
Peroxisome
DCA-CoA
ACOX
Lipogenesis
LCFA
ACOT3
ACOX
H202
LBP/DBP
MCFA
ACOT5
ACOT
Acetyl CoA
Malonyl CoA
ACC1
ACSI
Adipic
Suberic
Sebacic
ACOT8
MC-DCA-CoA
Acetyl CoA
Acetate
ACSII
Peripheral tissue
Succinyl CoA
ACOT4
Succinate (Propionate)
Gluconeogenesis

that these drugs can have unexpected adverse or beneficial effects in disease management. This is apparent by rofecoxib-mediated cardiovascular events where inhibition of COX2 results in a 120-fold increase in the blood level of 20-HETE and a dramatic increase in coagulation due to 20-HETE platelet aggregation and vessel vasoconstriction (Liu et al., 2010). It is of interest that polymorphism in the CYP4F2 gene has been associated with the idiosyncratic difficulties in warfarin therapy through CYP4F2's ability to metabolize and inactivate vitamin K2 necessary for activation of blood

Figure 5.4 ***CYP4A and CYP2C P450s function in liver eicosanoid and fatty acid metabolism.*** Fatty acids are delivered to hepatocytes for catabolism by the mitochondria or peroxisome β-oxidation systems or are used for the synthesis and export of triglycerides (TG), phospholipid (PL), cholesterol esters (CE), as very low density lipoprotein (VLDL) particles. Medium- and short-chain-length fatty acids are transported as acylcarnitine derivatives into mitochondria for complete β-oxidation to CO_2. Long- and very–long-chain fatty acid CoA esters as well as eicosanoids are transported into the peroxisome where they undergo chain-shortening reactions and then transported as acylcarnitine derivatives to the mitochondria for complete oxidation or as free fatty acids by thioesterase 3, or 5 (ACOT3, 5). Under normal conditions, (5–10%) fatty acids are converted to dicarboxylic acids CoA (DCA-CoA) by CYP4A or CYP4F ω-hydroxylation. Starvation induces the ω-oxidation of fatty acids by 40% through PPARα induction of CYP4A genes. The dicarboxylic acids, adipyl-CoA (C6), sebacyl-CoA (C8) or suberyl-CoA (C10) are exported from peroxisome by the action of acyl-CoA thioesterase 8 (ACOT8), or when ACOT8 is inhibited by free CoASH, the FF-CoA is converted to succinyl-CoA, which is exported as succinate after removal of CoA by ACOT4. Succinate can function as an anaplerotic intermediate in the mitochondria for gluconeogenesis during starvation and excess acetate produced from acetyl-CoA can be used by peripheral tissues after conversion to acetyl-CoA by acetate-CoA synthetase (ACSII). In the fed state, long-chain fatty acids (LCFA) and very-long-chain fatty acids (VLCFA) are metabolized by peroxisome β-oxidation to shorter chain products, which can be incorporated into phospholipids, cholesterol esters or triglycerides for export as VLDS and stored in peripheral tissues. Excessive acetate in the cytosol is converted to acetyl-CoA by hepatocyte acetate-CoA synthetase I (ACSI) and then converted to malonyl-CoA for fatty acid and cholesterol synthesis by ACC1. CYP4F gene induction by insulin is mediated by SREBP and saturated fatty acid-CoA activation of hepatocyte nuclear factor 4α (HNF4α). In the presence of excessive fatty acids, insulin will activate SREBP1c increasing the synthesis of stearoyl-CoA desaturase 1 (SCD-1) and converting palmitic (C16:0) and stearic (C18:0) acids to palmitoleate (C16:1) and oleic (C18:1), respectively, which are stored as triglycerides. Both CYP4 P450 and SCD-1 use cytochrome b_5 and cytochrome b_5 reductase in their catalytic cycle. The induction of CYP4A genes by high-fat-diet and an increase in SCD-1 with suppression of CYP4F genes may prevent the liver from lipotoxicity at the expense of steatosis and development of steatohepatitis. ACOX, acyl-CoA oxidase; LBP, L-bifunctional protein; DBP, D-bifunctional protein; TG, triglycerides; PL, phospholipid; CE, cholesterol ester; WE, wax ester. (For color version of this fi gure, the reader is referred to the online version of this book).

factors (Bejarano-Achache et al., 2012; Pavani et al., 2012). These data indicate that channeling of eicosanoids to different pathways and genetic polymorphisms of CYP genes have a significant role in the effectiveness of drug targeted to eicosanoid metabolism in the treatment of inflammation and metabolic diseases.

3.2. Catabolism of Prostaglandins and Leukotrienes

The inactivation and catabolism of bioactive eicosanoids are important in the inhibition of the inflammatory response and diseases associated with MetS. The design of effective therapies in the treatment of metabolic diseases of eicosanoid metabolism can lead to unanticipated consequences, which are evident in the development of sEH inhibitors used to prevent the inactivation of vasodilatory EET to diHETEs (Panigrahy et al., 2012). EETs are autocrine and paracrine mediators of vasorelaxation in the cardiovascular and renal systems through activated EET receptor-mediated stimulation of Gαs and subsequent activation of adenylate cyclase. The production of cAMP activates protein kinase, leading to activation of potassium BK_{Ca++} and K_{ATP} K^+ efflux and hyperpolarization causing vasodilation, with activation of eNOS and inhibition of NF-κβ. Therefore, sEH inhibitors are in clinical trials as antihypertensive agents and are being evaluated for use in the treatment of diabetes, stroke, dyslipidemia, immunological disorders, vascular remodeling, chronic obstructive pulmonary disease, and atherosclerosis (Shen, 2010). However, using both genetic and pharmacological methods to control the endogenous EET levels in vivo, it was recently demonstrated that EETs are critical for both primary tumor growth and metastasis in several mouse models of cancer (Panigrahy et al., 2012). Increased EETs elevate VEGF receptor 2 and serum VEGF levels, and decreased endogenous levels of angiogenesis inhibitor, thrombospondin. This study raises serious concerns about the chronic use of sEH inhibitors in CVD, which may have adverse effects in cancer patients.

Both EETs and hepoxilin of the 12-LOX pathway are metabolized and inactivated by sEH. EETs have antiinflammatory properties, while hepoxilin are proinflammatory, which suggests that sEH has a central role in modulating the inflammatory response. Although sEH is the major pathway for catabolism for 14,15-EET and is less important in catabolism of 11,12-, 8,9-, or 5,6-EET (Imig, 2012), when sEH is low or inhibited, elongation and peroxisome β-oxidation produce inactive 16-carbon epoxy FA (Spector et al., 2004). Equally, EETs can be ω-hydroxylated by CYP4A and CYP4F and directed to peroxisome for β-oxidation

(Fig. 5.4). Many human cancers have increased expression of CYP epoxygenase that promotes angiogenesis and cancer metastasis. Therefore inhibition of CYP2C and CYP2J2 gene expression or activation of sEH may be an effective treatment for cancer. PPARα ligands downregulate CYP epoxygenase gene expression and thus limit tumor metastasis (Bozza et al., 2011). In addition, CYP4X1 that is highly expressed in tumors and human breast cancer has been shown to metabolize AA to 8, 9-, and 14,15-EET (Stark et al., 2008). Both angiogenesis and inflammation are independent of stromal processes that exert substantial influence on tumor growth and metastasis. EET stimulation of angiogenesis and suppression of inflammation most likely signals through independent pathways either by activation of the unidentified EET receptors (Chen, Wang, et al., 2011) or through activation of PPARα or PPARγ receptors. CYP4A and CYP4F-mediated 20-HETE is a proinflammatory eicosanoid that stimulates production of PGE_2; the chemokines IL8, IL13, IL14; and TNFα cytokine (Ishizuka et al., 2008). 20-HETE activates NF-κβ, promoting cell survival, and activates the MAPK/ERK pathways that stimulate endothelial angiogenesis, proliferation and migration (Guo et al., 2007).

The major enzyme in the catabolism of PGs is 15-hydroxy prostaglandin dehydrogenase (15-PGDH). 15-PGDH also oxidizes and inactivates LXA_4, 15-HETE, and 12-HHT of the TX synthase pathway (Tai, 2011). The 15-keto metabolites are further metabolized by NAD(P)H-dependent 15-keto prostaglandinΔ_{13}reductase (13-PGR) to produce the inactive 13,14 dihydro-15-keto metabolites. 13-PGR is also known as LTB_4-12-hydroxy dehydrogenase and catalyzes the oxidation of 12(R) hydroxyl group of LTB_4 to inactive 12-keto-LTB_4. Thus both 15-PGDH and 13-PGR are involved in the catabolic inactivation of PGs and LTs. Activation of these enzymes ameliorates inflammation by inactivation of LTB_4 and induction of COX2 production of PGE_2. In cancer cells, the upregulation of COX2 by IL1β, and TNFα decreases the expression of 15-PGH. In contrast, adenovirus overexpression of 15-PGH induces COX2 upregulation in a dose-dependent manner that is not dependent on the catalytic activity of 15-PGH. It is of interest that the ω3-PUFA increases the expression of 15-PGDH and suppresses COX2 in hepatocellular carcinoma. However, the molecular mechanism responsible for this reciprocal regulation of COX2 and anti-inflammatory 15-PGDH remains to be determined (Tai, 2011). Several studies have shown that NSAIDs induce the expression of antiinflammatory 15-PGDH, while this induction was inhibited by proinflammatory cytokine induction of COX2. The induction of 15-PGDH

by PPARγ agonists suggests the possibility that NSAID inhibition of COX2 may channel AA to other eicosanoid pathways producing activators of PPARγ and 15-PGDH expression (Hazra et al., 2007). The parallel regulation of 15-PGDH with the prostaglandin transporter (PGT) in inactivating PGE_2 signaling represents an important target in the treatment of metabolic disease with an inflammatory etiology.

The major pathway for the inactivation and catabolism of hydroxyl eicosanoids is through peroxisome β-oxidation and conjugation with glucuronic acid. CYP4 ω-hydroxylase P450 begin the process of catabolism by ω-hydroxylation of eicosanoids to alcohols that are further metabolized to the corresponding aldehydes by alcohol dehydrogenase (ADH4) followed by FA aldehyde dehydrogenase (ALDH32a/FALD) to produce dicarboxylic acids that are solely metabolized by peroxisome β-oxidation (Fig. 5.4). In human neutrophils, CYP4F3a metabolizes and inactivates the proinflammatory chemotactic eicosanoid LTB_4. The eicosanoid dicarboxylic acids are chain-shortened by peroxisome β-oxidation and these short-chain products are fully oxidized to CO_2 by mitochondrial β-oxidation (Wanders, Ferdinandusse, Brites, & Kemp, 2010). A major difference between PG and LT catabolism by peroxisome β-oxidation is that PGs are chain-shortened from the C1 carboxyl group after CoA activation, while LTs and HETEs are metabolized from the ω-terminal carboxyl end. The peroxisome β-oxidation of eicosanoids and excessive FAs seen in MetS diseases employ the same pathway of CYP4 ω-hydroxylation, ADH4, and FALD to produce dicarboxylic acids. The metabolic catabolism of eicosanoid and excess FFA by the ω-hydroxylase cascade is critical for the termination of bioactive eicosanoids and the prevention of lipotoxicity observed in both MetS and NAFLD.

3.3. Transport and Transcellular Metabolism of Eicosanoids

In order eicosanoids to elicit their paracrine and autocrine effects, eicosanoids must be exported from the cell by a series of efflux transporters that have overlapping substrate specificities with drug metabolites and endogenous toxic biochemicals. Of equal importance is the uptake or influx of eicosanoids for transcellular synthesis by the recipient cells to bioactive eicosanoids that either activate HNRs or are metabolized and inactivated by eicosanoid catabolic enzymes. In general, the efflux transporters are members of the ABC transport superfamily, and the influx transporters are members of the SLC family. The ABC efflux and SLC influx transporters function to maintain optimal cell concentrations of nutrients, antioxidants,

and signaling molecules. The inactivation of PGE_2 occurs through a two-step process where the MRP4-ABCC4 mediates the efflux of PGE_2, PGD_2 and $PGF_2\alpha$, while importation into the recipient cell for inactivation occurs by the PGT, which is a member of the organic anion SLC carrier organic anion transporter (OAT)/SLC22. Once inside the recipient cell, the PG is inactivated by sequential metabolism by 15-PGDH and 13-PGR.

There are 49 human ABC transporters that function in the efflux of cholesterol, PLs, drugs, nucleosides, peptides, organic anions, and eicosanoids. Several diseases have been associated with defective ABC transporter, including familial intrahepatic cholestasis (ABCB4 and ABCB11), cystic fibrosis (ABCC7), type II diabetes (ABCC8), hyperbilirubinemia (ABCC2), adrenoleukodystrophy (ABCD1), and dyslipidemia syndrome (ABCA1). However, presently, no disease has been associated with eicosanoid efflux transporters. The importance of these transporters in eicosanoid metabolism in inflammatory disease is apparent from their induction by cytokines and regulation by NSAIDs and the inability of defective inflammatory response in MRP1-null mice to efflux LTC_4 (Leier, Jedlitschky, Buchholz, & Keppler, 1994). There are eight MRP/ABC transporters that efflux eicosanoids: MRP1/ABCC1 (LTC_4, LTD_4, LTE_4), MRP2/ABCC2 (LTC_4, PGA_2), MRP3/ABCC3 (LTC_4), MRP4/ABCC4 (PGE_2, $PGF_2\alpha$, PGD_2, TXB_2, LTB_4, LTC4), MRP5/ABCC5 (cyclic nucleotides), MRP6/ABCC6 (LTC_4), MRP7 (LTC_4) and MRP8 (LTC_4) (van de Ven et al., 2009).

MRP1/ABCC1 is a high-affinity transporter of reduced glutathione (GSH)-conjugated eicosanoids and is also active in transport of drugs and toxic agents, which can lead to GSH depletion and oxidative stress (Henkin et al., 2012). MRP2/ABCC2 functions in the transport of bilirubin, LTC_4, and glucuronide-conjugated acetaminophen, and defects in this transporter are responsible for hyperbilirubinemia in Dubin-Johnson patients. MRP is inhibited by MK-571 and Montelukast, both LT receptor antagonists. This transporter also has a significant role in PC transport into the enterohepatic circulation. MPR3/ABCC3 is upregulated in cholestastic disease and functions to secrete glucuronidated biochemicals into the hepatic sinusoids. MRP4/ABCC4 is the major eicosanoid efflux pump for prostanoids and GSH-conjugated LTB_4 and LTC_4 that are inhibited by NSAIDs. MRP4 mediates the efflux of several endogenous metabolites that have a critical role in signaling pathways involved in differentiation, pain, and inflammation. Because NSAIDs are strong inhibitors of MRP4 mediated efflux of PGs and LT, this may be another mechanism responsible for the antiinflammatory effects of NSAIDs. In MRP4-null mice, the decreased plasma

PG levels were correlated with an increase in the toleration of inflammatory pain (Lin et al., 2008). MRP5/ABCC5 is expressed in most tissues and similar to MRP4 transports cyclic nucleotides and thus functions as an OAT. It is rather surprising that the efflux transporter for LTA_4 has not been identified considering its important role in transcellular synthesis of LTs. It is likely that multiple transports function in LTA_4 transport similar to those of LTC_4. The wide range of immune cells expressing efflux transporters of eicosanoids clearly reveal their role in the orchestration of an effective immune response and significant undetermined role of immune cell involvement in metabolic diseases.

In contrast to ABC efflux transporter of eicosanoids, the SLC protein family members are responsible for the uptake and influx of eicosanoids in recipient cells (Wu et al., 2011). The first identified eicosanoid influx transporter was the PG transporter PGT/SLCO2A1, which is an OAT. Additional members of the OAT family (OATP/SLCO/SLC22) have been identified in the cellular uptake of eicosanoids and display similarities with FA transporter SLC27 members (Emami Riedmaier et al., 2012). The cellular uptake of eicosanoids by SLC22 transporters may interact with the SLC27 FA transport to connect energy needs with an inflammatory response (Niemi et al., 2011). During inflammation, increased circulating levels of PGE_2 trigger the PG transporter to normalize PGE_2 levels through 15-PGDH and 13-PGR PGE_2 catabolism. This homeostatic mechanism is also achieved by the newly identified OAT-PGE_2, $PGF_2\alpha$, and PGD_2 transporter of the SLC22 family in the kidney proximal tubules where PGE_2 activation of PG receptor EP4 increases PGE_2 catabolism and activation of the OAT-PG transporter. To date, three influx transporters of eicosanoids have been identified, OATP2A/PGT/SCLC21A2, OATP2B/SLC21A9, and OATP4A/SLC21A2, suggesting that members of the FA transporter family SLC21 may be important in the selective influx, catabolism or transcellular metabolism of eicosanoids. The transcellular synthesis of eicosanoids involves a donor cell generating an eicosanoid intermediate that is released from donor cells by MRP and chaperoned to recipient cells by albumin, liposomes or FABP to prevent hydrolytic attack by water, and taken up by SLC transporters.

A number of unanswered questions remain regarding eicosanoid efflux and influx transporters as to their role in modulating the inflammatory response in insulin resistance, diabetes, obesity, and NAFLD. However, their importance in metabolic disease is evident from reduced expression of the efflux transporter in models of NAFLD (Lickteig et al., 2007)

and the recent report of a FA efflux transporter in adipocytes (Henkin et al., 2012). Central to understanding the role of eicosanoid transporters in metabolic disease is the characterization of transporter involved in transcellular eicosanoid metabolism and regulatory roles of eicosanoids for the activity of the FA transporter in liver, pancreas, adipose and muscle tissue (Folco & Murphy, 2006).

3.4. Molecular Mechanism of Eicosanoid Regulation through GPCRs

Eicosanoids elicit their effect through paracrine, autocrine, and intracrine mechanisms by either the activation of extracellular G-protein-coupled eicosanoid receptors (GPCR), transcellular synthesis, or eicosanoid activation of transcription factors of the HNR family (Table 5.2). Even though these mechanisms have been well-studied with regard to inflammation and immune response, the role in the control of tissue response to eicosanoids in the regulation of metabolic pathways in MetS and NAFLD has only recently been studied. Excessive lipid droplets (LDs) accumulation of lipids in cells (steatosis) leads to the formation of LDs consisting of neutral lipids as TAG surrounded by a PL monolayer with unique FA composition that includes eicosanoids and a distinct set of proteins comprising many of the eicosanoid metabolic enzymes. In leukocytes, a portion of AA is stored in LD triglycerides where it is believed that mobilization of TAG AA by adipose triglyceride lipase (ATGL) replenishes lipid body arachidonyl-phospholipid through activation of LD-associated $cPLA_2$ providing AA for local eicosanoid synthesis (Bozza et al., 2011). Many of the eicosanoid biosynthetic enzymes are associated with LD and actively produce PGE_2, LTB_4, and LTC_4 on inflammatory stimulus. How eicosanoids exit the LD seems to be resolved with the identification of MRP14 transporter that is involved in AA transport and shuttling of uSFA to LD membrane (Vogl et al., 2007). Several studies have shown a correlation between eicosanoid synthesis by COX and LOX and an increase in the LD biogenesis (Bozza & Viola, 2010). In addition, PGD_2 activation of DP1 and DP2 receptors on eosinophils increases cytoplasmic LD biogenesis and synthesis of LTC_4. Eicosanoid synthesis and LD biogenesis are regulated by distinct signaling pathways through activation of DP1 and DP2 PGD_2 receptors. Activation of DP2 does not increase LD biogenesis or eicosanoid synthesis, while DP1 activation increases LD biogenesis, and both are required for eicosanoid synthesis, indicating that these receptors coordinate the increase in LD biogenesis and LTC_4 synthesis (Mesquita-Santos et al., 2011).

Table 5.2 Mechanism of regulation of G-protein coupled receptors (GPCRs) and Nuclear hormone receptors (NHRs) by bioactive lipids)

Gene id	Nomenclature	Tissue	Regulation	Substrate ligand	Protein interaction	Function
FFAR1	GPR40	Pancreas	—	C10-C18, TZD	Gq	GSIS secretion
FFAR2	GPR43	Adipose	PPARγ	C2-C5	Gq,Gi	Inhibit lipolysis
FFAR3	GPR41	Intestine, adipose	—	C2-C4	Gi	GLP, Leptin secretion
	GPR84	MT	—	C9-C12	Gi	IL12-p40
	GPR119	Pancreas, intestine	—	LysoPC, 5-HEPE	Gs	GLP-2,PYY secretion
	GPR120	Colon, adipose	—	C10-C18, PUFA	Gq	GLP-1 secretion
EP1			—			
EP2	PGE_2 receptor	MT, CNS, T-B	—	PGE_2	Gq	$Ca2^{+}$↑, CVS, immune
EP3	PGE_2 receptor	MT, CNS, DC, E, F	—	PGE_2	Gs	cAMP↑, immune
EP4	PGE_2 receptor	MT, CNS,K, IM	—	PGE_2	GI,$G_{12/13}$	cAMP↓, fever
DP1	PGE_2 receptor	MT, DC, T,CAN	—	PGE_2	Gs,Gi	cAMP↑, inflammation
DP2	PGD_2 receptor	VSMC, BSMC, P	—	PGD_2, PGJ_2	Gs	cAMP↑, intestine
IP	PGD_2 receptor	Th2, MT, IM	—	PGD2, PGJ_2	Gi	cAMP↓,chemotaxis
FP	PGI_2 receptor	VSC, MT, IM	—	PGI_2	Gs,Gq	cAMP↑
TP	PGF_2 receptor	MT, K, L, IM	—	$PGF_2\alpha$	Gq	Ca^{2+}↑
BLT1	Thromboxane	MT, IM P, CNS	Sp1,AML1	TXA_2, isoprostanes	Gq	Ca^{2+}↑
BLT2	LTB_4 receptor	Myeloid, spleen	Sp1	LTB_4, 20-LTB_4,12-HETE	Gi	Chemotaxis, proliferation

cysLT1	LTB_4 receptor	Spleen, Liver,	AP-1,GATA	LTB_4,12-HETE, 20-LTB_4	Gi	Chemotaxis, proliferation
	LTD_4 receptor	myeloid	STAT6	$LTD_4 > LTC_4 > LTE_4$	Gq	SRS-A, inflammation
cysLT2	LTC_4 receptor	Leukocytes, S,L,I	STAT1,Jak	$LTC_4 = LTC_4 > LTE_4$	Gq	SRS-A, inflammation
ALX/FPR2	LXA_4 receptor	Leukocytes, S,B, heart	—	LXA_4, 15epiLXA_4,peptides	Gs,i,q	Antiinflammatory
LPA1-5	LPA receptor	MT, myeloid MT	—	Lysophosphatidic acid	Gi,Gq,$G_{12/13}$	CVS, proliferation
NR1C1	PPARα	Liver, kidney	FXRα	LTB_4, 8-HETE, EET, EPA,		B-oxidation
NR1C2	PPARβ	Muscle	—	DHA, CLA, PL, fibrates		Antiinflammatory
NR1C2	PPARγ		C/EBP	15-keto PGE_2,		β-oxidation, antiinflammatory
NR2A1	HNF4α	Muscle, adipocyte	—	PGI_2, 15-HETE,EPA, RA		Adipogenesis
NR1H3	LXRα	macrophage	—	15dPGJ_2, 15-HETE, EPA		Antiinflammatory
NR1H4	FXRα	Liver, MT	—	13-HODE, $PGF_2\alpha$,TZD		β-oxidation
NR5A2	LRH-1	Liver	—	Aminosalicylic acid		Antiinflammation
NR2B1	RXRα	Liver	—	Linoleic, saturate FA		Cholesterol sensor
NR1B1	RARα	Liver	LXRα	Oxysterol		Bile acid sensor

Continued

Table 5.2 Mechanism of regulation of G-protein coupled receptors (GPCRs) and Nuclear hormone receptors (NHRs) by bioactive lipids)—cont'd

Gene id	Nomenclature	Tissue	Regulation	Substrate ligand	Protein interaction	Function
NRA4	NAR4	MT	GPCR, TLRs	Bile acids		Antiinflammatory
NROB2	SHP	MT	FXRα	Phospholipids		Glucocorticoids
		MT		Cis-retinoic acid, DHA,		HNR partner
		MT		All-trans retinoic acid		Differentiation
				Unknown		β-adrenergic signal
				Retinoids?		β-oxidation
						Energy glucose homeostasis

AML1, acute myeloid leukemia1; B, basophils; CVS, cardiovascular system; Th2-CRTH2, chemoattractant receptor homologous receptor; CNS, central nervous system; BSM, bronchial smooth muscle; CLA, conjugated linoleic; DC, dendritic cell; E, endothelia; F, fibroblast; GSIS, glucose-stimulated insulin secretion; GLP, glucagon like peptide 1; K, kidney; L, lung; I, intestine; IM, immune cells; MT, multiple tissues; PYY, pancreatic YY peptide; SRSA, slow-reacting substance of anaphylaxis; S, spleen; STAT, signal transducer activator transcription; TZD, thiazolidinedione; TLR, Toll-like receptors.
This table includes receptor's primary tissue of expression, regulation, ligand or substrate activation and primary function in target tissue.

Because LD formation in pancreas and liver increases insulin resistance, hepatic steatosis, and obesity, it is believed that LD may be the primary source of metabolic persistent inflammation in the pathology of MetS. There are few studies to address the issue of LD formation and inflammation in different organs and cell types and whether LD biogenesis and increased eicosanoid synthesis initiate and amplify the lipid actacoid-cytokine-chemokine cascade. LD formation has been strongly associated with muscle insulin resistance in T2DM (Bosma et al., 2012). Muscle insulin resistance is due to SNARE protein that controls the fusion of LDs, which is necessary for the progression of microvesicular steatosis to macrovesicular steatois in the liver. In muscle, SNARE protein associates with the plasma membrane and assists in insulin-mediated translocation of Glut4 from the cytosol. Excessive muscle TAG shifts the SNARE protein from the plasma membrane to LD formation resulting in insulin resistance (Bostrom et al., 2007). Thus, increased LD biogenesis in MetS with elevated tissue synthesis of inflammatory eicosanoids not only promotes recruitment of immune cells but also activates plasma membrane and NHRs in the control of lipid and carbohydrate metabolism through autocrine, paracrine, and intracrine mechanisms.

A broad range of lipid mediators, including FA discussed previously, PGs, TXs, HETE, oxo-ETT, LTs, and lysoPLs act on GPCRs. The plethora of GPCRs, about 376, provide the cell a unique mechanism to both maintain metabolic homeostasis and respond to a changing environment by specific and selective agonists and antagonists. About 50% of the currently prescribed drugs target GPCRs. Although lipid mediators are considered a small group of GPCR activators, they have been proved to be an important viable target in the treatment of inflammation and MetS.

3.4.1. Fatty Acid Receptors in Regulation of Metabolism

The extracellular effects of FFA signal through a group of GCPR (Ichimura et al., 2009). These receptors include FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41), GPR84, and GPR120 that recognize FAs with different chain length and degree of unsaturation to initiate both antiinflammatory and anti-obesity effects. These SCFA, medium-chain FA and LCFA receptors function in diabetes and inflammation. Glucagon-like peptide-1(GPL-1) secretion in the intestine and pancreatic insulin secretion are mediated by activation of the GPR120 and GPR40 receptors by ω3-PUFA and C18-20 uSFAs, respectively while GPR40 is also activated by TZD. The divergent response of pancreas to FFAs, where acute exposure

stimulates insulin secretion, while chronic exposure impairs insulin secretion, is revealed in GPR40-null mice that display insulinemia and, insulin resistance, but protection from steatosis, hyperglycemia and hypertriglyceridemia (Steneberg et al., 2005). GPR40 is activated by LCFAs (C_{12}-C_{16}) that induce gastrointestinal cells to secrete GLP-1. GRP119 is activated by LCFAs and lysoPLs in the intestine where activation stimulates intestinal K-cells to secrete glucose insulin-trophic peptide and L-cells to discharge GPL-1 incretins. In the pancreas, release of GLP-1 is glucose independent, but GPR40, and FA dependent. GPR43 and GPR41 are activated by SCFAs (propionate, butyrate, pentanoate) that regulate FA and glucose homeostasis in adipose tissue and intestine. SCFA mediated stimulation of GPR43 reduces serum FFAs by inhibiting adipose lipolytic activity and thus has an important role in the lipid profile of MetS patients. GPR43 is prominently expressed in leukocytes and may have a role in leukocyte activation of inflammation in hyperlipidemia. GPR43-null mice fed an HF diet display a lean phenotype with increased energy expenditure and improved glucose tolerance, and the absence of this receptor in adipocytes increases energy expenditure, while absence in macrophages prevents inflammation. GPR41 is also expressed in adipose tissue where it is believed to regulate leptin production since receptor activation increases serum leptin levels. GPR84 is a medium-chain FFA receptor that is highly induced in leukocytes in inflammation. Thus, both GPR84 and GPR43 in leukocytes link FA metabolism to inflammation and immune cell activation. GPR120, usually activated by ω3-PUFAs stimulates intestinal secretion of GLP-1 and increases Glut4 transporter expression in adipose tissue. Importantly, GPR120 attenuates inflammation by inhibiting TLR2, TLR4, and TNFα receptor-mediated inflammation in macrophages (Oh & Olefsky, 2012; Talukdar et al., 2011). Overall, GPRCs regulated by FFAs are direct sensors of nutrients in the extracellular environment that mediate secretion and possibly production of peptide hormones. These GPRs function as important modulators of inflammation and immune system function thus further linking lipid metabolism to inflammation (Oh da & Olefsky, 2012).

3.4.2. Eicosanoid G-protein-Coupled Receptors

Prostanoid receptors consist of five types that bind a diverse array of prostanoids including PGD_2 (DP1 and DP2), PGE_2 (EP1-4), $PGF_2\alpha$ (FP), PGD_2 (CRTH2), PGI_2 (IP), and TXA_2 (TP). Although numerous studies have revealed the role of these receptors in inflammation and immune regulation,

only few studies have suggest these receptors in the control of metabolism like the FFA receptors (Hirata & Narumiya, 2011). The prostanoid receptors can be classified by their cellular response as relaxant receptors that increase cAMP through Gαs (DP1,EP2, EP4, and IP), or activation of Ca^{2+} mobilization and contractile response by Gαq (EP1,FP,TP) while inhibitory receptors connected to Gαi (EP3). The DP1 PGD_2 receptor mediates smooth muscle cell vasodilation, inhibition of platelet aggregation and activation of mast cell in allergic inflammation. The DP2 receptor and chemoattractant receptor homolog (CRTH2) in Th2 cells promote immune cell migration in inflammation, and induce basophil and esosinophils to secrete IL4, IL5, and IL13 cytokines. EP1 receptors (PGE_2) signal through Gαq to promote tissue edema and pain. EP1-null mice show a significant reduction in systolic blood pressure and thus antagonist can be used to treat hypertension of MetS. An intriguing role of EP1 receptor in the central nervous system is its activation during stress-induced release of glucocorticoids similar to fever generation by hypothalamic-pituitary-adrenal axis stimulation through activation by both EP1 and EP3 receptors (Furuhashi & Hotamisligil, 2008). PGE_2 antiinflammatory effects are mediated through EP1/EP4 suppression of TNFα production, enhanced IL10 synthesis, and inhibition of T-cell mitogenesis with enhancement of Th1 cell differentiation and Th17 cell expansion. Blocking both the EP1/EP4 receptors decreases Th1 and Th17 cell accumulation in lymph nodes and suppresses progression of autoimmune encephalomyelitis (Esaki et al., 2010). The EP3 receptor mediates fever generation since EP3-null mice show no fever in response to inflammatory stimuli. The EP4 receptor in the vasculature acts as a potent vasodilator and cooperates with EP2 in inflammation. EP4-null mice develop severe colitis, which can be mimicked by EP4 antagonist in wild-type mice (Kabashima et al., 2002). The IP (prostacyclin) is localized in endothelial cells and its activation has potent antithrombotic and vasodilator effects that oppose the effect of TXA_2 since IP-null mice show accelerated atherogenesis in ApoE-deficient mice (Kobayashi et al., 2004). PGI_2 has potent antiinflammatory and immunosuppressive effects on Th2-mediated inflammation through IP suppression of dendritic cell activation and maturation of T cells (Zhou et al., 2007) while promoting Th1 cell differentiation. TP (TXA_2) induces platelet aggregation and smooth muscle contraction that opposes the prostacyclin vascular effects. TXA_2 produced in dendritic cells activates T-cell TP receptors that inhibit dendritic cell immune response while activating inflammation. FP ($PGF_2\alpha$) is coupled to Gαi that inhibits cAMP and Gαq, which mobilizes Ca^{2+} and has a functional role in lung fibrosis by

stimulating fibroblast proliferation and collagen production independent of TGFβ (Oga et al., 2009). It will be of importance to determine if activation of FP in hepatic stellate cells initiates the pathology of steatohepatitis to hepatic fibrosis in NAFLD.

The LT receptors consist of two classes, the LTB_4 receptors that are expressed in myeloid cells, endothelial and smooth muscle cells (BLT1), and liver (BLT2) and the cysteine LT receptors (cysLT1 and cysLT2). The BLT receptors are activated by LTB_4 and ω-hydroxylase CYP4 LTB_4 metabolites, 20-OH LTB_4 and 20-COOH LTB_4, and TX synthase-produced 12-HHT. BLT1 activation is coupled to Gαq-mediated Ca^{2+} mobilization and Gαi inhibition of cAMP production. BLT2 has a 20-fold weaker binding of LTB_4 ($K_d = 1–23$ nM) compared to BLT1 ($K_d = 0.15$ nM) but can be activated by 12(S) HETE, 15(S) HETE and 12-HHT. BLT2 is expressed in liver, ovary, leukocytes, macrophages and mast cells where it mediates chemotaxis similar to BLT1. Both BLT1 and BLT2 activate several kinases, including MAPK, involved in macrophage proliferation, and phosphatidyl kinase that induces Ca^{2+} mobilization and IL6 gene expression and NF-κβ DNA binding. LTB_4 is critical for the induction of the neutrophil respiratory burst in the release of myeloperoxidase, matrix metalloproteinase, elastase and α-defensins. Activation of BLT receptors in macrophages induces IL1β, IL6, and MCP-1 production, and chemotaxis. The presence of both BLT receptors in macrophages allows chemotaxis to occur over a wider range of LTB_4 concentrations. In T cells, BLT1 is the dominant chemotactic receptor that induces production of IL1, IL2, IL5 and interferon-γ, which promotes Th17 cell differentiation (Chen et al., 2009). In VSM cells, BLT1 activation initiates SMC migration and proliferation through activation of integrin signaling (Moraes, Assreuy, Canetti, & Barja-Fidalgo, 2010) as shown by congenic BLT1-null ApoE-null mice having a dramatic reduction in the number of atherosclerotic lesions (Heller et al., 2005). Endothelial BLT1 is believed to be responsible for release of vasoactive factors, while BLT2 activation is required for angiogenesis, suggesting a correlation between LTB_4 levels and function of endothelium. BLT1- and BLT2-null mice show reduced atherosclerosis and an attenuated response to inflammatory arthritis, while only BLT2-null mice have colitis due to disruption of the intestinal barrier (Nancey et al., 2011) that can be mimicked by 12-HHT antagonists. There has been only one study investigating the function of BLT receptors in MetS (Spite et al., 2011). In BLT1-null mice, there is a reduction of adipose tissue M1 macrophages (ATM) while antiinflammatory M2 ATM numbers increase, resulting in decreased expression of proinflammatory chemokines

and cytokines. Also, BLT1-null mice fed an HF diet are protected from systemic glucose intolerance and have decreased hepatic steatosis with reduced adipocyte and liver inflammation. It will be of interest to determine the phenotype of BLT2-null mice on an HF diet or on ApoE-null background.

There are two cysteine LT receptors cysLT1 and cysLT2 that are activated by the cysteine leukotrienes (LTC_4, LTD_4, and LTE_4); however, these receptors show difference in regulation by agonists and antagonists. CysLT receptors are important in asthma because of their prominent expression in eosinophils; however, these receptors are also expressed in monocytes, granulocytes, B cells, fibroblasts, myocytes, and plasma where their activation increases release of monocyte chemotactic protein (MCP-1) and its translocation to the nucleus during inflammation. Both receptors are believed to have different roles in inflammation with cysLT1 mediating the effects of acute inflammation, while cysLT2 functions in chronic inflammation and fibrosis. Activation of cysLTs in the vasculature causes vessel constriction, increase in vascular permeability and cardiac output. Thus blockage of receptor activation inhibits atherosclerotic lesion size and intimal hyperplasia. CysLTs have been implicated in liver disease, which includes hepatic inflammation, cholestasis, portal hypertension and hepatorenal syndrome. Inhibition of receptor activation has been found to be effective in the prevention of liver and intestine injury by reducing apoptosis and oxidative stress (Daglar et al., 2009) and improving hepatic fibrosis in cholestasis (El-Swefy & Hassanen, 2009).

The balance between activation of proinflammatory receptors (LT) and antiinflammatory (LX) is critical in maintaining tissue homeostasis. The antiinflammatory LT LXA_4 and LXB_4 synthesized by transcellular metabolism from LTA_4 have potent antiinflammatory and resolution abilities that signal through LXA_4 receptors. LXA_4 receptors (LX) are coupled to Gαi/o, reduce chemotaxis and induce phagocytosis of apoptotic neutrophils. Although the pathophysiological role of LXA_4 and LX receptor is suggested, their role in human disease has at best been a causal association (Serhan et al., 2007) and thus their role in MetS and NAFLD has not been investigated.

The deacylation and reacylation of PL by the Land's cycle produces lysoPLs that are precursors to LPA, PAF and endocannabinoids. LysoPLC can be converted back to PL by the action of lysophospholipid:acyltransferase (LPLAT). LPA is a water-soluble PL that activates six currently known GPCRs for LPA that couple to Gαi, Gαq, and Gα12/13 (Lin et al., 2010). The strong binding of LPA to LPA receptors in the nanomolar range strongly

implicates their role in physiological functions. LPA in CVD appears to have a protective role in preventing hypoxia-induced ischemia by activation of PI3/AKT and ERK pathways, while inducing Src-mediated contraction. However, LPA receptors induce intimal hyperplasia, VSMC migration and proliferation, endothelium dysfunction, LDL uptake and monocyte recruitment and adhesion. Overall, LPA has both beneficial effects on I/R injury and adverse effects on the development of atherosclerosis. In the liver, LPA activates hepatic stellate cells, leading to collagen deposition in the extracellular matrix as well as induction of hepatocyte proliferation, which contribute to hepatic cirrhosis (Watanabe et al., 2007). Similar to cysLT receptors the LPA receptors function during the chronic stages of inflammation; however, their role in the progression of NAFLD has not been studied. Furthermore, although the elusive cytochrome epoxygenase EET receptor has not been identified, a recent report has provided conclusive evidence that this receptor mediates many of the physiological effects of EETs (Chen, Falck, Manthati, Jat, & Campbell, 2011).

To date, 30 GPCRs for lipid mediators have been identified and studied (Nakamura & Shimizu, 2011) with particular emphasis on FFA receptors in the control of lipid and carbohydrate metabolism and eicosanoid metabolism in inflammation and immune cell activation. Hopefully, future studies will identify the cross-talk between these FFAs and eicosanoid receptors in inflammation and intermediary metabolism in diabetes, obesity, hyperlipidemia, hypertriglyceridemia, and NAFLD.

3.4.3. Eicosanoid Regulation of Nuclear Hormone Receptors

The original purpose of defining MetS as a constellation of metabolic alterations of insulin resistance, dyslipidemia, hypertension, hypertriglyceridemia and obesity was to define the risk factors that contribute to CVD. The recent pandemic of obesity in the Western population has led to the identification of NAFLD as a disease with many symptoms of MetS. The prevalence of NAFLD in the Western population ranges from 1 to 36% with 90% of obese patients and 70% of type II diabetic patients strongly indicating that NAFLD is a strong predictor of MetS. With the realization that increased influx of free FA was due to alterations in lipid and carbohydrate metabolism controlled by NHRs, it became imperative to identify NHR agonists and antagonists that control FA and carbohydrate oxidation and decrease lipogenesis. The identification of NHR drug agonists that ameliorated some symptoms of insulin resistance and obesity (e.g. PPARγ-TZD agonists) led to the realization that

endogenous ligands may be altered thereby leading to MetS associated diseases (Table 5.2). Thus a hunt for endogenous ligands that regulate these orphan NHRs started and continues to of utmost importance in understanding and treatment of NAFLD and MetS. This quest has led to the search for lipid molecules that regulate NHR since lipidemia is a constant in both diseases. Whether eicosanoids are true physiologically important NHR ligands for many orphan NHRs has yet to be resolved through rigorous analytical methods, including in vivo co-localization of eicosanoids with NHR on target genes and testing in eicosanoid enzyme knockout mice. One dilemma in using eicosanoids as physiologic ligands is that even though NHR activation occurs in the eicosanoid nanomolar range, in vitro micromolar or greater levels must be used to observe a response, in vivo. However, the association of several eicosanoid metabolic enzymes with the nuclear envelope and FABP or ACBP transportation into the nucleus can increase eicosanoids levels high enough to activate NHR activation.

The NHR family is the largest group of transcriptional regulators in humans consisting of 48 members that include steroid homodimer receptors: androgen, estrogen, glucocorticoid (GR), mineralocorticoid, and progesterone; nuclear receptor heterodimers with retinoic-X-receptor (RXR); retinoic acid (RAR), thyroid, and vitamin D the partially unknown ligand orphan receptors: farnesoid-X-receptor (FXR), liver-X-receptor, pregnane-X-receptor (PXR), PPAR, estrogen related receptor, HNF receptors, liver-related homolog (LRH), and RAR-related orphan receptor (ROR); and lastly true orphan receptors Rev-erb, short heterodimer partner (SHP), and chicken upstream promoter transcription factor.

The NHRs most prominently involved in the control of metabolism and collectively known as metabolic sensors are PPARα, PPARβ, PPARγ, LXRα, FXRα, PXRα, constitutive androstane receptor (CAR), RXRα, and HNF4α. The PPARs are the most studied NHRs in regard to lipid activation by FFAs and eicosanoids. PPARα (NR1C1) is expressed in tissues with a high rate of FA oxidation, liver, kidney, intestine, and BATs. PPARβ (NR1C2) is expressed in adipose, skeletal muscle and several other tissues and has a fundamental role in cellular processes. PPARγ (NR1C3) is abundantly expressed in WAT, BAT, and liver where it promotes glucose uptake, lipid storage, adipocyte differentiation and maintenance. The PPARγ2 variant is most prominently expressed in adipose and muscle tissues, while PPARγ1 is broadly expressed in many tissues. A variety of

eicosanoids and PLs have been identified as PPAR ligands, connecting inflammation with the control of carbohydrate and lipid metabolism in in the development NAFLD and MetS (Harmon et al., 2011; Wahli & Michalik, 2012).

PPARα (NR1C1) is activated by uSFAs, LTB_4, 8(S)-HETE, 8,9-EET, 11,12-EET, 15-HETE, PLs and the synthetic fibrate drugs that reduce serum triglycerides. Activation of PPARα increases the transcription of genes involved in β-oxidation and has antiinflammatory effects. It is apparent that proinflammatory eicosanoids mediate their effects through activation of plasma membrane GPCRs, while these same proinflammatory eicosanoids induce an antiinflammatory response by activation of PPARα, PPARβ, and PPARγ. Activation of PPARα has several antiinflammatory properties that include induction of peroxisome β-oxidation and inactivation of eicosanoids (Narala et al., 2010), induction of I-κβ that blocks NF-κβ transcription of proinflammatory genes, increased expression of soluble interleukin-1 receptor antagonist (Stienstra et al., 2007), as well as transrepression of proinflammatory transcription NF-κβ, activator protein, and nuclear factor of T cell (Poulsen et al., 2012). PPARα also interacts with the GR that mediates an increase in cortisol levels, which that is believed to be responsible for hyperinsulinemia observed in sepsis (Ahmed et al., 2012). PPARα has a central role in metabolism and inflammation which is based on the observation of upregulation of TLR2 and TLR4 in the adipose tissue of PPARα-null mice that exhibit hepatic steatosis after fasting (Wahli & Michalik, 2012). PPARα expression in macrophages modulates cholesterol trafficking and inhibits local vascular inflammation by channeling excess FA to β-oxidation or TAG storage rather than the production of ceramides and DAG that are believed to be instrumental culprits in muscle insulin resistance.

PPARβ (NRIC2) is also activated by uSFA, VLDL constituents, and products of oxidized 4-hydroxy-2-nonenal and 4-hydroxydodeca-2E, 6Z-dienal, and is weakly activated by FAs. PPARβ antiinflammatory effects are evident by its inhibition of NF-κβ activation and transcriptional induction of anti-inflammatory corepressor B-cell lymphoma G, induction of angiopoietin-related protein 4, and increased expression of TGF-β. In adipocytes, PPARβ has a central role in signaling between ATM and adipocytes where it promotes M2 macrophage phenotype over the M1 ATM that is proinflammatory. Activation of PPARβ reduces atherosclerosis in LDLR-null mice by decreasing expression of MCP-1, vascular adhesion molecules,

and TNFα. In muscle, PPARβ channels palmitic acid to TAG accumulation and mitochondrial β-oxidation rather than production of lipids that initiate inflammation and insulin resistance. In cardiomyoctes, PPARβ inhibits palmitate and LPS-induced inflammation by transrepression of NF-κβ (Alvarez-Guardia et al., 2011).

PPARγ (NR1C3) is activated by uSFAs, oxidized FA, 9-HODE, 13-HODE, 15-HETE, 13-oxo-ODE, 15-deoxy$\Delta^{12,14}$ prostaglandinJ2, oxo-LDL, LPA, farnesyl phosphate, 15-keto-PGE_2, and decanoic acid, while both $PGF_2\alpha$ and cyclic PA are antagonists. PPARγ is activated by the synthetic antidiabetic TZDs that reduce serum hyperglycemia and promote adipocyte differentiation. PPARγ inhibits inflammation by repressing NF-κβ, and macrophage and T-cell expression of pro-inflammatory cytokines of the innate immune response (Huang & Glass, 2010). Macrophages from PPARγ-null mice exacerbate metabolic disease by increasing hepatic, adipose, and muscle insulin resistance. Although PPARγ activation increases adipocyte differentiation that has a beneficial effect on increasing systemic insulin sensitivity and reducing hyperglycemia, its induction in the liver promotes hepatic steatosis, while deletion protects against HF-diet-induced hepatic steatosis (Moran-Salvador et al., 2011). The decrease in PPARα and increase in PPARγ expression in hepatic steatosis may be a protective mechanism to prevent liver fibrosis. PPARγ-null mice display muscle and macrophage hypercholesterolemia, leading to atherosclerosis, disruption of endothelial cell dysfunction, and endothelial cell proliferation (Qu et al., 2012).

All three PPARs are therapeutic targets for the treatment of metabolic diseases and are important drug targets to treat the chronically persistent inflammation associated with these diseases. Although fibrates that target PPARα have been very successful in the treatment of dyslipidemia, the PPARγ TZD drugs despite improving insulin sensitivity have a number of undesirable side effects including weight gain, edema, heart failure, and bone fractures. Because PPARγ isoforms are expressed in several tissues associated with MetS, such as adipose, liver, VSMC, endothelial cells and pancreas, it is a desirable target to treat MetS if we can dissociate the undesirable site effects from its efficacy in treating hyperglycemia and hyperlipidemia. Recently, 5-amino salicylic acid, an anti-inflammatory drug that inhibits PGH_2 synthase, NF-κβ, 15-LOX, and PLA_2, while it activates PPARγ, shows less side effects than TZDs and has greater antiinflammatory properties (Wahli & Michalik, 2012). Also, decanoic acid (C10) a medium-chain FA, activates PPARγ without

inducing adipogenesis, yet improves insulin sensitivity and is also a weak agonist for PPARβ and PPARα (Malapaka et al., 2012). Other HNRs are also activated by lipids, but unlike the PPARs, it is not known whether they are also targets for eicosanoid metabolites in the treatment of MetS.

The liver-related homolog-1 (LRH-1/NR5A2), which is a competence factor for SHP, FXR, PPARα, and LXRα. LRH-1 is activated by distinct PLs and has profound antiinflammatory action by induction of glucocorticoid synthesis and reduction in acute-phase protein synthesis of serum amyloid A, haptoglobin, fibrinogen, and inhibition of IL6 and IL1β (Venteclef et al., 2011). LXRα/NRIH3 is a cholesterol sensor that is induced by oxysterol, activating genes in reverse cholesterol transport and repressing proinflammatory cytokine gene expression, thus contributing to reduced atherosclerotic lesions. Unfortunately, LXR activation also increases lipogenesis and VLDL serum levels and decreases ApoA1 necessary for synthesis of HDL particles. LXR coordinates the regulation of neutrophil homeostasis the clearance of senescent neutrophils by antigen-presenting cells in peripheral tissues (Hong et al., 2012). In addition, LXRα inhibits expression of the acute-phase protein, C-reactive protein, through transrepression of proinflammatory transcription factors (Venteclef et al., 2011).

FXRα/nuclear family 4 subgroup A receptors (NR4H4) is the main bile acid sensor that when activated inhibits NF-κβ and profibrogenic collagen 1α. FXR inhibits TAG synthesis and VLDL export through the FXR-SHP cascade as well as inhibits HDL metabolism while inducing VLDL catabolism. PXR/NR1I2 and the CAR/NR1I3 are xenobiotic-activated receptors that promote hepatic lipid storage by decreasing FA β-oxidation. PXR promotes free FA uptake through CD36 induction and thus induces lipogenesis, while CAR regulates serum TAG levels and CAR-null mice are protected from hepatic steatosis. Orphan receptor SHP/NR0B2 is a downstream target of FXR, has no DNA-binding domain. HNF4α/NR4A1 is a master regulator of lipid metabolism and mutations in this HNR lead to maturity onset of diabetes. This orphan receptor ligand is believed to be linoleic acid; however, this FA does not influence HNF4α transcriptional activity (Yuan, et al., 2009).

The orphan NR4A subgroup of receptors include Nur77 (NR4A1), Nurr1 (NR4A2), and Nor-1 (NR4A3); all are associated with lipid and carbohydrate metabolism in muscle, liver, and both WAT and BAT through NR4A activation of β-adrenergic signaling (Pearen & Muscat, 2010). This establishes a link between FA metabolism and eicosanoid

pathways. Although NR4A receptors have no known natural ligands, these receptors are key molecular switches linking inflammation to metabolism. Their bulky ligand-binding domain and regulation by PGE_2 make them a target for eicosanoids being their possible endogenous agonist (Mohan et al., 2012). NR4A2/NURR1 act as monomers to transactivate target genes. NR4A2 is activated by COX2-produced PGE_2 that activates the EP1 receptor, leading to induction of FA β-oxidation (Holla et al., 2011). It is especially interesting that NR4A members regulate the expression of FXRα, RXRα, SREBP-1 and PPAR coactivators, PCG1α and PCG1β.

Nuclear hormone receptors have a central role in macrophages' and dendritic cells' ability to sense their lipid environment through eicosanoid and lipid agonists that regulate NHRs, resulting in expression of proinflammatory M1 or anti-inflammatory M2 phenotype (Nagy et al., 2012). To deorphanize these NHRs, we need to identify their true endogenous ligands by characterization of receptor–ligand affinities, determination of cellular concentrations of ligands by liquid chromatography tandem mass spectrometry, and also in vivo colocalization of ligand and receptor with a defined physiological response.

4. EICOSANOIDS IN SEPSIS AND DRUG METABOLISM

4.1. Links between Sepsis and MetS

Sepsis or septicemia is defined as systemic inflammatory response syndrome (SIRS) that affects over 750,000 patients annually in the United states with a mortality rate of over 30% (Angus et al., 2001). SIRS is a constellation of both metabolic and inflammatory derangements that ultimately lead to multiple organ failure by increased circulating levels of proinflammatory cytokines, cortisol, acute-phase proteins, and apoptotic immune cells. SIRS patients display many of the symptoms of MetS largely due to excessive serum cortisol and adrenocorticotropic hormone (ACTH), which are similar to those of Cushing syndrome patients who have insulin resistance with hyper-insulinemia, hyperlipidemia, hypertriglyceridemia, and hypertension (Macfarlane et al., 2008). The progression of local sepsis to systemic sepsis is initiated by overactivation of the lipid autocoid-cytokine-chemokine cascade, leading to overstimulation of immune system and dramatic suppression of drug- and eicosanoid-metabolizing P450s. This situation makes the treatment of sepsis a balance between effective management and the possibility of inadvertent

drug toxicity (Seeley et al., 2012). The importance of eicosanoid in the initiation and progression of sepsis is apparent considering clinical trials of sepsis patients with ω3-PUFAs had a 20% decrease in mortality and reduction in serum enzymes, cortisol and ACTH that correlated with a massive increase in EPA-derived LTs (Grimm et al., 2006). Although parenteral and enteral ω3-PUFAs appear to preserve immune function and reduce inflammation, the role of eicosanoids in the progression and management of sepsis has not been extensively studied. Of equal importance is the question how eicosanoids regulate cortisol levels and what effects they have on adrenal gland.

The characterization of a patient's serum profile may have a potential utility in staging of sepsis, which is very difficult due to the heterogeneity of septic patients with respect to sites of infection, type and virulence of pathogens, and comorbidities including liver disease, cancer, age, and environmental variables (Seeley et al., 2012). In septic patients, PGE_2 and 11-HETE have recently been identified as differentiating eicosanoid metabolites between healthy subjects and sepsis patients (Bruegel et al., 2012). In septic patients, the levels of PGE_2 and 11-HETE are reduced by 80% with a decreased expression of inducible COX2 but not mPGES-1, suggesting that antiinflammatory suppression of the Th-1-mediated immune response is not functional in septic patients. Thus the infusion of PGE_2, which mediates suppression of Th-1 and activation of adaptive Th-2 immune response, may have a promise with ω3-PUFAs in the management of sepsis (Nicolete et al., 2008).

It is known that activation of the immune system by inflammation during sepsis leads to a dramatic alteration and repression of the drug-metabolizing enzymes with reduced expression of selective CYP genes. Adverse drug reaction (ADR) is a serious human health problem caused by idiosyncratic effects of drugs during their therapeutic use in the treatment of diseases (Deng et al., 2009). Idiosyncratic adverse drug reactions (IADR) are caused by accumulation of toxic drugs and endogenous biochemical metabolites during inflammation. It may not be a mere coincidence that IADRs are the most common cause of liver failure in sepsis. The COX inhibitor, diclofenac, inhibits COX1 and COX2 enzymes in inflammation as well as being a substrate and inhibitor of CYP2C8 and CYP2C9 that produce antiinflammatory EETs. A nontoxic dose of LPS given to rats rendered a nontoxic dose of diclofenac injurious to the liver (Deng et al., 2006), suggesting that inflammation is a pivotal factor

in diclofenac-induced IADR. It is likely that the competition of COX isoforms and CYP2C8 or CYP2C9 for the synthesis of prostanoids and EETs, respectively, is altered by diclofenac in inflammation, where its normal metabolism by CYP2C8 is reduced or completely inhibited leading to diversion of AA from prostanoid and EET synthesis to proinflammatory 5-LOX and 12-LOX pathways (Fig. 5.2). This would explain the beneficial antiinflammatory effects of PGE_2 infusion in the treatment of sepsis. The decreased CYP2C19 enzymatic activity in critically ill patients would result in decreased NSAID metabolism and inhibition of COX-mediated production of PGE_2, being replaced by AA metabolism to proinflammatory LTs. Thus, overexpression of CYP epoxygenase (CYP2C8, CYP2J2) attenuates NF-κB-dependent vascular inflammatory response in vivo and may inhibit chronic inflammation in sepsis patients (Deng et al., 2011).

The cause of IADR in sepsis and inflammation may be suppression of cytochrome P450 activity by unknown mechanisms. It has been suggested that the increased synthesis of acute phase response competes with CYP synthesis in the liver or that inhibition of cytokine mediates inhibition of NHR that regulates the CYPs gene expression. Although numerous studies have shown that selective CYPs are downregulated during inflammation and sepsis, there are few studies on how eicosanoids regulate CYP expression. Kupffer cells, hepatic macrophages, have been shown to mediate the decreased expression and activity of CYP1A1, CYP1A2, and CYP2E1 in the inflammatory response (Kim et al., 2011), and decreased expression of the major liver drug-metabolizing CYP3A4 and CYP2C isoform's mRNA and enzymatic activities has been reported in mice exposed to LPS (Moriya et al., 2012). Furthermore, suppression of hepatic CYP2C and CYP2J mRNA after LPS induction of inflammation decreased levels of EETs and also decreased CYP4A12 and CYP3F13 production of 20-HETE (Theken et al., 2011). The repression of EET and 20-HETE formation by CYP2 and CYP4 P450s, respectively, was also evident in lung, and kidney, but no difference in EET + DHET or 20-HETE was observed in the heart. Although this study needs confirmation, it demonstrates that activation of the innate immune response by inflammation alters the expression of eicosanoid-metabolizing CYPs, leading to reduced synthesis of bioactive EETs and 20-HETE with reduced metabolism of therapeutic drugs that can lead to IADRs.

5. EICOSANOIDS AND METS DISEASES

The epidemics of obesity, T2DM, and atherosclerosis in MetS are increasing yearly worldwide. The constellation of diseases associated with MetS, insulin resistance, hypertriglyceridemia, hyperlipidemia, hypertension, and obesity are largely attributed to derangements in lipid and carbohydrate metabolism. The rate of NAFLD is increasing in the United States with 34% of the population displaying many of the symptoms of MetS, thus making NAFLD an additional characteristic of MetS (Anderson & Borlak, 2008). However, the mechanism in the pathophysiology of fat accumulation in the liver (hepatic steatosis) and how fatty liver communicates with other tissues in the diseases of MetS are not fully understood. Here we will briefly discuss the role of eicosanoids in the control of carbohydrate and lipid metabolism in tissues having a functional role in MetS and NAFLD and how inflammatory effects on CYP drug and eicosanoid metabolism lead to subclinical organ dysfunction in NAFLD, obesity, insulin resistance, dyslipidemia, and hypertension. Our intent in this section is to provide suggestive evidence for eicosanoids functioning as an important link between inflammation and intermediary metabolism and therefore an unrealized therapeutic target to treat MetS.

5.1. Eicosanoids in NAFLD and Obesity

Hepatic steatosis refers to the intracellular accumulation of lipids and the formation of LD in the cytoplasm of hepatocytes that can progress from simple microvesicular steatosis to macrovesicular steatosis and eventually fatty liver inflammation (steatohepatitis), which is also known as NASH. The excessive accumulation of lipids leads to increase in lipid peroxidation by reactive oxygen species (ROS), ultimately leading to immune cell recruitment and infiltration into damaged tissue. The persistent subclinical inflammation is caused by activation of the lipid autocoid-cytokine-chemokine cascade with the eventual activation of hepatic stellate cells with secretion of collagen for increased hepatic fibrosis that can progress to hepatic cirrhosis. The role of eicosanoids in NAFLD until recently has been largely explored with respect to Kupffer cell-mediated cytokine-chemokine-related disease progression.

The importance of eicosanoids in NAFLD is evident from metabolic analysis of eicosanoid-metabolizing enzyme knockout mice and the analysis of the lipidome of NAFLD patients. In patients with NAFLD or NASH, analysis of serum plasma lipidome revealed an increase in monounsaturated

fatty acid (MUFA), C16:1n7 and C18:1n9, with an increase in the saturation index (uSFA to SFA) (Puri et al., 2009). In addition, linoleic acid (C18:2n6) decreased with an increase in dihomo-γ-linolenic acid (C20:3n6) and a decrease of antiinflammatory DHA and EPA in PE and PC PLs. Furthermore, there is a stepwise increase in LOX metabolites (5-HETE, 8-HETE, 15-HETE and the nonenzymatic oxidation product HHT) with the progression of NAFLD to NASH. Analysis of oxidized lipids in the plasma of patients with NASH showed increased levels of HETE, HODE, and oxo-octadecadienoic acid (oxoODE) that strongly correlated with free-radical-mediated oxidation of linoleic acid to 9- and 13-HODE and 9,13-oxoODE products that increase with the progression from steatosis, to steatohepatic and fibrosis in patients with NAFLD (Feldstein et al., 2010). Presently, we do not know the source of ROS that initiates progression of NAFLD to NASH (Fig. 5.2). Recent data with CYP2e1-null mice and the corresponding wild-type mice suggest that CYP2E1 is likely to provide ROS in high-fat induced NASH development (Abdelmegeed et al., 2012).

Thus perturbation in hepatocyte lipid metabolism and the accumulation of intrahepatic lipids and LD is the first hit leading to steatosis, while intrahepatic ROS from either altered metabolism of excessive FFA or through recruitment of immune cells constitute the second hit in the progression of steatosis to steatohepatitis (Day & James, 1998). With the excessive lipids in LDs that serve as a source of eicosanoid biosynthesis (Bozza et al., 2011), it is possible that selective eicosanoid metabolites from LD may serve as not only initiators of the lipid-cytokine-chemokine cascade but also regulators of lipid metabolism in hepatic steatosis. Both obesity and insulin resistance are strongly associated with NAFLD with increased insulin resistance in adipocytes, leading to an increase in adipose triglyceride lipase (ATGL) and HSL activity, resulting in hydrolysis of adipose TAG and elevation in serum FFAs. Free FA uptake by liver is mediated by FATPs that channel FAs to β-oxidation, production of VLDL for transport to peripheral tissues, or the formation for LDs when excessive lipids are taken up by the liver. Excessive FAs stimulate ACSL3 translocation to nascent LDs (Poppelreuther et al., 2012). Proteomic analysis of LD proteins revealed the association of ADSL1, ACSL3, and AA-CoA-activating ACSL4 with hepatic LDs (Hodges & Wu, 2010). The attenuation of ACSL3 expression correlates with PL class switching, while in ACSL3-null mice, there was a reduced incorporation of FFAs in LDs of human Huh7 hepatoma cells (Yao & Ye, 2008). The association of lysoPC-acyltransferase with

LDs suggests that these organelles are in dynamic equilibrium (Moessinger et al., 2011). Recently, a direct role of LD in ACSL4 esterification of AA-CoA and COX2 activation has been reported in the metastatic potential of breast cancer and LT production. LD ACSL4 may serve a similar function in hepatic steatosis by providing AA for the synthesis of inflammatory 5-LOX metabolites, since ob/ob mice treated with 5-LOX inhibitors show restored microsomal transfer protein (MTP) and VLDL secretion and were protected from hepatic steatosis.

In hepatic steatosis, ACSL5 expression is elevated and channels FAs to LDs thus competing with FA β-oxidation and VLDL transport pathways. Excessive FAs are highly toxic to hepatocytes. Thus, to prevent lipotoxicity excess, FAs are stored as TAG in LD or metabolized by induction of peroxisomal FA β-oxidation. Peroxisomal FA β-oxidation produces chain-shorted FAs that are completely oxidized by mitochondrial β-oxidation. However, FA mitochondria transport by CPT-1 in hepatic steatosis is inhibited by lipogenic production of the CPT-1 inhibitor malonyl-CoA. Thus LD size expansion through storage of excess FAs as TAG is an adaptive mechanism to prevent lipotoxicity. Central to production of TAG is the synthesis of monounsaturated palmitic and stearic acids by SCD-1. SCD1 and ACC1 are transcriptionally induced by SREBP1 during lipogenesis where SCD-1 regulates the partitioning of monounsaturated and SFAs in steatosis. The critical role of SCD-1 in FA partitioning in steatosis and steatohepatitis is evident in SCD-1-null mice fed a methionine-choline (MCD) diet, which reduces PC and PE levels. These mice have decreased body weight and hepatic steatosis but markedly increased hepatocellular apoptosis, liver injury, and fibrosis (Li et al., 2009) compared to wild-type SCD +/+ mice fed a MUFA diet that prevented MCD-induced injury. This study indicates the critical role of SCD1 in partitioning of excess FFA into MUFA for either transport as VLDL or storage at TAGs in LDs in hepatic steatosis. Thus SCD1-null mice accumulate pro-inflammatory saturated FFAs, which leads to hepatic steatohepatitis. Critical for SCD1 enzymatic activity is the role of cytochrome b_5 reductase and cytochrome b_5 not only in the desaturation of SFA but also in the desaturation and elongase-mediated production of AA. Both cytochrome b_5 and reductase also have a critical role in the coupling of electron transport from NADPH cytochrome P450 oxidioreductase (OR) to cytochrome P450 in the metabolism of drugs and production of EETs and 20-HETE by CYP2 and CYP4 P450 isoforms. Both cytochrome b_5 and reductase are human obesity susceptibility genes (Rankinen et al., 2006).

Increased intrahepatic SFAs are known to cause lipotoxicity and insulin resistance in the liver, and SCD1 and ACC1 control the ability of the liver to provide nutrition to peripheral tissues through gluconeogenesis, ketogenesis, and VLDL secretion. SFAs differentially affect these key regulatory enzymes causing liver insulin resistance by an unresolved paradox of selective insulin resistance where insulin is able to activate lipogenesis through IRS-1, but not IRS-2-mediated suppression of gluconeogenesis thus causing hyperglycemia (Brown & Goldstein, 2008). Normally, activation of the IR results in phosphorylation and activation of IRS-1 toward SREBP1c increase in lipogenesis, while IRS-2 activation mediates phosphorylation of FOXO-1 and exclusion from the nucleus where it activates the transcription of genes involved in gluconeogenesis. It has been reported that SFAs activate JNK1, which somehow preferentially phosphorylates and inactivates IRS-2 and thus prevents inhibition of gluconeogenesis. However, this idea has been challenged by the fact that hepatocytes from JNK1-null mice show glucose intolerance, insulin resistance and steatosis (Sabio et al., 2009). The mechanism of increased lipogenesis in hepatic insulin resistance may be mediated by ChREBP activation of SREBP-1 transcription in concert with PPARγ2 induction in liver by HF diet and the transcriptional coactivator PGC-1β. Recent evidence indicates that SFAs can induce c-Src clustering with plasma membrane subdomains leading to JNK activation, while MUFA blocks this partitioning and activation of JNK (Holzer et al., 2011). It will be of importance to determine if s-Src is associated with LDs and whether LD membranes have higher concentrations of palmitic and AA in their lipid bilayers.

Besides the channeling of excess FA to LD for TAG synthesis, the peroxisome β-oxidation system has a critically significant role in the prevention of FA-induced lipotoxicity. The peroxisome L-bifunctional enzyme enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (EHHADH) is critical in the metabolism of long-chain dicarboxylic acids and has recently been identified as a candidate gene in NAFLD (Banasik et al., 2011) and a novel regulatory gene of the P450 system (Yang et al., 2010) (Fig. 5.4). At least three genes CYP4A190, CYP4A14, and aldehyde dehydrogenase, Aldh3a2, are involved in the EHHADH peroxisome β-oxidation regulatory network (Houten et al., 2012). In steatosis-resistant A/J mice fed an HF diet all these genes are induced with increased metabolism of long-chain dicarboxylic acids (Hall et al., 2010). A bioinformatics approach identified EHHADH as a candidate gene associated with T2DM, obesity, and glucose intolerance. These data strongly implicate peroxisome β-oxidation as a protective

regulatory system to prevent hepatic steatosis and also suggest that the FA ω-hydroxylase CYP4A and CYP4F P450s have a central role in producing dicarboxylic acids from excessive FAs, and an undefined role in the ω-hydroxylation of eicosanoids in NAFLD. Recently, a functional variant of human CYP4F2V433M was associated with all the features of MetS except glucose (Fava et al., 2012). This variant has previously been associated with hypertension and stroke due to the reduced ability to metabolize particular substrates, including vitamin K in warfarin therapy. However, this variant is as efficient in the ω-hydroxylation of LTB_4 as the CYP4F2 wild-type gene indicating that other eicosanoids or LCFA substrates may be inefficient substrates (Stec et al., 2007).

We had previously reported that ob/ob mice fed an HF diet induces the expression of CYP4A genes and protein, while CYP4F expression was reduced (Hardwick, 2008; Zhang & Hardwick, 2000). Moreover, we have observed that insulin induces the expression of the CYP4F2 in human hepatocytes. The CYP4F2 gene is regulated by SREBP-1 (Hsu et al., 2007) and thus may function to metabolize excess FAs in the peroxisome for further metabolism as dicarboxylic acids. The increased expression of CYP4A in hepatic steatosis and steatohepatitis implies that they have a functionally unidentified role in enhancing lipotoxicity as revealed by their dramatic induction in CYP2E1-null mice with an increase in ROS and lipid peroxidation upon exposure to the MCD diet (Leclercq et al., 2000). Increased production of dicarboxylic acid during steatosis by CYP4A isoforms can impair mitochondria function by dissipation of mitochondrial proton gradient and uncoupling of oxidative phosphorylation. In addition, the uncoupling of the P450 catalytic cycle is a major source of microsome ROS (Narasimhulu, 2007) that led to the identification of CYP2E1 as a source of ROS-induced lipid peroxidation in HF-induced steatohepatitis (Abdelmegeed et al., 2012). However, CYP2E1 is not induced in human liver during steatosis or steatohepatitis and in fact decreases with disease progression, while CYP4A11 levels increase suggesting that CYP4A isoforms may be the major source of ROS in human hepatic steatosis. The recent identification of mitochondrial targeting of CYP2E1 may not only account for CYP2E1 decrease in microsomes but also may account for increase in ROS formation in mitochondria in NAFLD (Knockaert et al., 2011). CYP2E1 can metabolize FAs at the ω-1 position and CYP4A is able to ω-hydroxylate medium-chain FAs. However, as chain length increases, CYP4A11 loses specificity and begins to ω-1 hydroxylate longer chain FAs (Hardwick et al., 2009). It is not known whether this change in FA

hydroxylation with different chain length FAs increases uncoupling of the P4504A catalytic cycle and increases ROS production. In addition, we do not know if cytochrome b_5 increases coupling of the P450 catalytic cycle and reduces ROS formation. Since cytochrome b_5 and reductase are increased in obesity and both enzymes are used for both P450 catalytic cycle and desaturase reactions, sequestering cytochrome b_5 by SCD-1 may lead to increased uncoupling of the P450 catalytic cycle in the presence of FA excess. Normally, the induction of CYP4A genes during fasting provides both gluconeogenic precursors and acetate to meet the energy needs of peripheral tissues (Fig. 5.4); however, their induction during NAFLD may increase hyperglycemia, shuttle acetate for lipogenesis and increase ROS by FA uncoupling of the P450 catalytic cycle.

It is not known whether CYP4F2 gene is differentially regulated by SFAs or MUFAs in patients with NAFLD; however, we have evidence that both CYP4F2 and CYP2E1 are repressed in patients with NAFLD, while the neutrophil CYP4F3 expression increases with progression of disease. The CYP4F2 gene expression is downregulated by peroxisome proliferators and fibrate drugs (Zhang et al., 2000), induced by retinoic acid, and increased by lovastatin through SREBP-2 (Hsu et al., 2007). The induction of CYP4F2 and SCD-1 genes by insulin in primary hepatocytes suggests that their differential regulation in insulin resistance may be a determining factor in hepatic steatosis. Thus the induction of CYP4F2 gene expression by retinoic acid, which has been shown to ameliorate steatosis (Ashla et al., 2010), may decrease the formation and storage of TAG in the liver, and prevent recruitment of immune cells by increased metabolism of LTB_4 and thus the progression of steatosis to steatohepatitis. Although there are numerous reports of FA induction of cytokine and chemokine production in hepatocytes, we need to further study the function of LTs in the activation of the lipid autocoid-cytokine-chemokine cascade in recruitment of immune cells to the liver in NAFLD. The association of the liver CYP4F2 and neutrophil CYP4F3 gene in inflammatory celiac disease establishes a connection between neutrophil recruitment to the established Th1 innate immune response in disease patients (Curley et al., 2006). In human HepG2 cells treated with saturated FAs (C16:0-C18:0) or unsaturated FAs (C18:1-C18:2) at subphysiological levels from 50 to 200uM, there was a greater induction of CYP4A11 mRNA with SFAs and a marked repression of CYP4F2 by all FAs with no change in CYP4F3b expression (Madec et al., 2011). In addition, in ApoE-null mice fed an HF diet there was a repression of epoxygenase activity and induction of CYP4 ω-hydroxylase activity,

leading to a significant increase in the 20-HETE/EET+DHET ratio in kidney compared to no change in the liver (Theken et al., 2012). Furthermore, kidney podocytes exposed to diabetic high glucose concentrations showed increased ROS formation associated with sequential upregulation of CYP4A, 20-HETE and NADPH oxidase activity (Eid et al., 2009), leading to podocyte apoptosis as seen in diabetic patients. Furthermore, the effects of high glucose on induction of NOX mRNA, protein, and activity were blocked by CYP4A inhibitors and mimicked by 20-HETE, indicating that CYP4A induction initiates podocyte apoptosis and diabetic proteinuria.

The importance of drug metabolism in hepatic steatosis is apparent in NADPH cytochrome P450 reductase (OR)-null mice that develop hepatomegaly and fatty liver due to induction of C36 FA transporter, SCD-1, and CYPs involved in bile acid synthesis (CYP7A1), steroid (CYP51), and retinoic acid metabolism (CYP26a1) with a repression of FA oxidation genes CPT1a, EHHADH and CYP4A10 (Weng, et al., 2005). There is increasing evidence that drug-metabolizing enzymes, consisting of phase I CYP, phase II conjugation, and phase III transporters, are differentially expressed in hepatic steatosis; however, their role in the initiation and progression of NAFLD requires further study especially in the context of eicosanoid metabolism, conjugation and transport in the immunological response to excess FAs (Christensen & Hermann, 2012). In NAFLD, the mRNA and protein for major drug-metabolizing CYP isoforms such as CYP1A2, 2D6, 2E1, 2C19 and 3A4 are decreased with NAFLD progression, while CYP2A6, 2B6 and epoxygenase CYP2C9 increase (Fisher et al., 2009). The decreased expression of CYP correlated with increased expression of pro-inflammatory expression of TNFα, and IL-1β possibly by lipid autocoids. In human hepatocytes treated with 1 mM FFA mixture (2:1 ratio of oleate and palmitate), there was a repressed expression of CYP1A2, 2A6, 2B6, 2E1, 3A4 and the epoxygenase CYP2C9 (Donato et al., 2007). These data indicate that FFAs but not cytokines or chemokines control the expression of these CYPs, and that induction of CYP2C9 in vivo, but repression by FFA in vitro, indicates a differential regulation of this epoxygenase in the progression of NAFLD to NASH.

Numerous clinical studies have reported significant changes in drug pharmacokinetics in patients with inflammation, cancer, and sepsis, which is largely due to the differential response of drug-metabolizing enzymes to cytokine- and chemokine-mediated repression. This variable depression of selective CYP can increase patient drug exposure up to 400%, resulting

in IADRs, therefore it will be important to determine how NAFLD and NASH influence the pharmacokinetics of drugs, especially drugs targeting eicosanoid metabolism in inflammation, especially in regard to alterations in phase I, II, and III enzyme expression and activity. The role of eicosanoids in the progression of NAFLD, and their role in inflammation have been recently studied in NASH. However, recent studies have also identified a key role of eicosanoids in the regulation of intermediary metabolism in the progression of NAFLD by the use of global knockout mice of eicosanoid-metabolizing enzymes, eicosanoid metabolism inhibitors, and congenic strains developed on mouse models of MetS.

Early studies on the cause of NAFLD focused on the immunological aspects of inflammation in NASH where IL-6, PGE_2 and TNFα produced in Kupffer cells directly influence lipid metabolism in hepatocytes (Enomoto et al., 2000). In alcoholic steatohepatitis, increases in TAG correlated with PGE_2 levels, indicating that Kupffer cell activation by ethanol enhances PGE_2-mediated effects on hepatic lipogenesis. In addition, COX2-derived PGE_2 was shown to inhibit TGFβ1-mediated induction of collagen synthesis by activated stellate cells, thus inhibiting fibrosis (Hui et al., 2004). It is apparent that PGE_2 has divergent effects by inducing steatosis by inhibiting steatohepatitis possibly by a recent identified mechanism of nonsubstrate FAs binding the allosteric site of COX1, inhibiting catalytic activity, but stimulating COX2 activity and PGE_2 production (Zou et al., 2012). These results indicate that COX2 is not only induced by cytokines in inflammation but also by FAs in hepatic steatosis. The progression of steatosis to steatohepatitis is largely mediated by eicosanoids of the LOX pathway. During acute inflammation, 5-LOX and 12/15-LOX pathways are activated and resolution of inflammation is mediated by the transcellular metabolism of hepatocyte 15-HETE by Kupffer cell 5-LOX to produce antiinflammatory LXA_4 and LXB_4, which inhibit chemotaxis, selectin and integrin endothelial immune cell adhesion as well as transmigration of neutrophils across the endothelium. It is likely that autocoid lipids mediate the coordinate regulation of metabolic activities in Kupffer cells and hepatocytes through unknown mechanisms that need to be delineated in greater detail in relation to the role of prostanoid and LT pathways in NAFLD.

Disruption of the eicosanoid-metabolizing enzymes has provided insight into the role of eicosanoids in MetS and NAFLD with respect to alterations in carbohydrate and lipid metabolism. In PLA_2IVA-null mice fed an HF diet hepatic steatosis, was reduced with smaller adipocytes caused by reduced serum PGE_2 that has lipogenic effect on adipocytes. In global knockout of

5-LOX, hepatic necroinflammation, hepatic immune cell infiltration, hepatocyte ballooning and serum alanine aminotransferase (ALT) levels were significantly reduced with a marked reduction in hepatic steatosis (Titos et al., 2010). These symptoms were due to a marked reduction of lipogenic gene expression determined by ingenuity pathway analyses that are affected by loss of 5-LOX activity. Congenic mice with double knockout of both ApoE and 5-LOX showed reduced hepatic inflammation and serum ALT due to reduction in serum levels of inflammatory cytokines and chemokines (Martinez-Clemente, Ferre, Gonzalez-Periz, et al., 2010) thus supporting a role of eicosanoids in the initiation of the lipid autocoid-cytokine-chemokine cascade. Metabolically, ApoE/5-LOX double knockout mice are remarkably insulin sensitive because of upregulation of PPARγ, IRS-1 and serum adiponectin levels, while JNK1 activity is reduced. Furthermore, the 12/15-LOX Alox15 gene is upregulated in ApoE-null mice that spontaneously develop hyperlipidemia (Martinez-Clemente et al., 2010). However, in ApoE/12/15-LOX congenic mice, there is a reduction in serum ALT, hepatic steatosis, inflammation and macrophage infiltration into the liver. In Alox15-null mice fed an HF diet, increased insulin resistance was attenuated and there was an upregulation of IRS-2 and AMPK and inhibition of JNK1 kinase activity, resulting in attenuated hepatocellular injury (Czaja, 2010). Of equal importance, when COX2 knockout mice, which develop atherosclerosis due to the reduced synthesis of PGI_2, are crossed with ApoE-null mice, the congenic strain develop accelerated atherogenesis with lesions exhibiting excessive leukocyte infiltration and upregulation of vascular adhesion molecules. These data indicate that diversion of AA to LT pathway exacerbates atherogenesis and thus suggests that chronic administration of NSAIDs may increase cardiovascular risk. It is apparent that global knockout of COX and/or LOX gene activity influences both carbohydrate and lipid metabolism in NAFLD. It is imperative that floxed mice for eicosanoid enzymes be developed to identify the tissue-specific role of eicosanoids in the regulation of metabolism in NAFLD.

5.2. Visceral and Subcutaneous WAT

Adipose tissue consists of several depots located in two body compartments: under the skin (subcutaneous depot) and in the body trunk (visceral depot). The main cell of adipose organ is the adipocyte, which can be white, located in WAT, or brown adipocytes located in BAT. Adipose tissue functions in fuel metabolism, lactation, thermogenesis and immune response and thus represents a dynamic organ in the maintenance of whole-body homeostasis.

Brown adipoctyes function primarily in energy utilization by thermogenesis and thus contain large mitochondria with extensive cristae and elevated levels of uncoupling protein 1 that functions to dissipate the mitochondrial proton motive gradient in the generation of heat. In contrast, WAT stores energy in small adipocytes in the visceral adipose tissue (VAT) or in large adipocytes in the subcutaneous adipose tissue (SAT). WAT express leptin adiponectin, and S100B, and associated with WAT is a lymphocyte population that expresses the leptin receptor, suggesting a relationship that may have importance in the energy requirement of an immunological response (Moro et al., 2010).

The adipose tissue stores display remarkable organ plasticity where WAT can be stimulated to transdifferentiate into BAT after adrenergic stimulus or prolonged exposure to cold. Recently, the adipokine, Irisin, released from exercising muscle has been shown to increase WAT conversion to BAT (Bostrom et al., 2012). The transdifferentiation of WAT to BAT occurs through a number of mechanisms and thus has important implications in MetS since animals with more BAT are resistant to obesity and Type II diabetes, while animals without BAT are prone to obesity and T2DM (Cinti, 2012). Thus exercise or treatment with beta-3-adrenoreceptor agonist, irisin or adiponectin may be important new avenues for the treatment of obesity and T2DM.

Not only the amount of BAT and WAT but also the amount of SAT and VAT have an important role in obesity and T2D. Excessive consumption of fat and carbohydrates increase WAT by adipocyte hypertrophy, differentiation of preadipocyte, and transdifferentiation of BAT by TGFβ. Gender, age, and environmental and genetic factors influence bodily distribution of fat. Since VAT is highly correlated with the development of obesity, T2D, and recently NAFLD, lean patients with abdominal obesity and increased VAT have an increased incidence of NASH (Filik, 2011). It is proposed that fat redistribution to VAT when SAT become full or restricted initiates the development of MetS. Support for this theory comes from studies in which patients treated with PPARγ agonist that promote preadipocyte expansion of SAT, attenuates NAFLD and markedly improves symptoms of MetS. Insight into the mechanism of VAT and SAT ectopic fat redistribution may be stress induced (Mittendorfer, 2011). In patients with NAFLD, expression of 11β-hydroxylsteroid dehydrogenase type I, which converts inactive cortisol to active corticosterone, is elevated in VAT, but not in SAT (Candia et al., 2012). Furthermore, rats fed an HF diet and implanted with corticosterone pellets develop severe insulin resistance, hyperinsulinemia,

hyperglycemia, and hypertriglyceridemia, characteristics of MetS that are not evident in either HF diet or corticosterone-treated rats (D'Souza et al., 2012).

As VAT expands, the adipocytes become hypertrophic and produce a signal that attracts macrophage of MI proinflammatory phenotype. These macrophages encompass the dying VAT adipocyte forming crownlike structures (CLS). These CLS are more prevalent in VAT over SAT in obese individuals and these macrophages are often called Mac2 macrophages since they are immune reactive for galactose-specific lectin 3. The Mac2 macrophage phagocytoses the dying adipocyte and produces proinflammatory IL-6, TNFα, and IL-1α, which interferes with IR signaling. Obese mice and humans with VAT CLS have increased insulin resistance of peripheral tissues, while hypertrophic VAT that do not display CLS are insulin sensitive (Virtue & Vidal-Puig, 2010). Furthermore, VAT release of FFA is twofold higher than in SAT in humans with NAFLD suggesting a pathological role of VAT, but not SAT adipocyte lipolytic, function in MetS (Thorne et al., 2010). Many clinical and epidemiological studies indicate that VAT is directly associated with abdominal and liver fat content independent of total adipose mass, BMI and SAT (Hall et al., 2012; Hamdy et al., 2006). Many studies have found that VAT and liver fat are associated with MetS independently, such that VAT was more important in lower levels of obesity, while liver fat was associated with severe obesity and the development of MetS (Kim, Nalls, et al., 2011). These findings support the portal vein hypothesis in which FFAs, proinflammatory cytokines, and adipokines from VAT contribute to increased hepatic lipid stores and inflammatory cell recruitment with hepatic and peripheral tissue insulin resistance (Virtue & Vidal-Puig, 2010).

It is generally accepted that metabolic dysfunction arises from lipotoxicity caused by lipid intake that exceeds what an individual adipose tissue can store. Thus the adipose tissue expandability hypothesis suggests that each individual has a threshold for storage of lipids in either the SAT or VAT depots (Virtue & Vidal-Puig, 2010). However, it is uncertain how adipocyte dysfunction is initiated and how lipotoxicity in VAT leads to metabolic dysfunction, causing NAFLD and eventually MetS. Recent insight into the metabolic abnormalities associated with obesity and increased VAT depots have come from studies with lean and obese monozygotic twins and large cohort studies where elevated levels of β-hydroxybutyrate ketone bodies in serum were strongly associated with T2D (Gall et al., 2010). Furthermore, obese monozygotic twins have elevated levels of membrane PLs containing

PUFA with lower carbon number and increased double bonds than lean nonobese twins (Pietilainen et al., 2011), which is due to increased ELOV6 expression in VAT that is believed to function in membrane remodeling during adipocyte hypertrophy. Therefore, the increase in n-6 PUFAs may be an early metabolic event in VAT that initiates the production of proinflammatory eicosanoid lipid mediators that modulate events in both lipid storage and lipotoxicity (Du et al., 2012; Murphy, 2001).

The type of PUFA in adipose tissue has a dramatic effect on adipocyte metabolism, adipose dynamics, and inflammatory environment of adipose tissue. The ratio of n-6 and n-3 PUFA in VAT and its metabolism to either pro- or antiinflammatory eicosanoids can have a significant role in adipocyte differentiation, function, and the production of inflammatory chemokines and cytokines. PG of the J_2 series derived from AA has a dual effect in adipocytes by increasing adipogenesis through activation of PPARγ and activation of monocyte chemoattractant protein (MCP-1), during the maturation phase of adipogenesis (Hossain et al., 2012). The production of PGJ_2 from PGD_2 can be prevented by both selective and nonselective COX2 inhibitors, thereby inhibiting the maturation phase of adipogenesis and significantly suppressing the accumulation of adipose fat (Ghoshal et al., 2011). Furthermore, the dynamics of adipocyte remodeling are highly dependent on the ratio of n-6 and n-3 PUFA in adipocyte PLs. The n-6 AA/n3 EPA ratio is elevated in VAT over SAT of severely obese women with metabolic dysfunction, which was strongly negatively correlated with adiponectin levels (Caspar-Bauguil et al., 2012). In contrast, high EPA and DHA levels increased adiponectin mRNA and secreted protein in 3T3-Li adipocytes (Tishinsky, Ma, & Robinson, 2011). EPA inhibits TNFα-induced lipolysis by downregulating HSL and decreases ATGL protein in primary rat adipocytes, resulting in the inhibition of HF-diet-induced hyperglycemia and hyperinsulinemia (Kalupahana et al., 2011; Lorente-Cebrian et al., 2012).

Hypertrophy of VAT adipocyte is an initial step in adipocyte cell death and recruitment of Mac2 macrophage that leads to the formation of CLS. Hypoxia is a key regulatory of adipose tissue dysfunction and heme oxygenase-2 (HO-2) is an important regulator of physiological levels of ROS. HO-2 knockout mice show depletion of mesenchymal stem cell (MSC) adipocytes that result in increased adipogenesis and production of proinflammatory cytokines and decreased HO-1 activity and production of EETs (Burgess et al., 2012). In contrast, upregulation of HO-1 and CYP2J5 production of epoxyeicosatrienoic acid (EET) decreased MSC adipocyte

differentiation, increased adiponectin secretion and decreased proinflammatory cytokines, suggesting that HO-1 and EETs protect against adipocyte hypertrophy and ensuing MetS (Burgess, Vanella, Bellner, Gotlinger, et al., 2012; Burgess, Vanella, Bellner, Schwartzman, et al., 2012). Of the 263 secreted proteins from human adipocytes, of which 44 were identified as novel adipokines, HO-1 circulating levels and VAT tissue expression is significantly increased in obese subjects compared to lean controls (Lehr et al., 2012). HO-1 is involved in the reduction of oxidative stress and inflammation and is released by mature SAT in obese individuals. It is of interest that increased TNFα secretion by VAT downregulates HO-1 secretion, while HO-1 induction reduces WAT secretion of TNFα. These data suggest an important link between VAT and SAT in modulating ROS and inflammation. Soluble epoxide hydratase (sEH) levels increase during adipocyte cell differentiation and are markedly elevated in obese mice (De Taeye et al., 2010). Inhibition of sEH leads to an increase in EETs in VAT of mice fed a HF high-fructose diet. Increased EET levels result in a reduction in serum leptin, decrease in VAT, decreased calorie intake, increase in metabolic rate and significant weight loss (do Carmo et al., 2012).

Chronic low-grade persistent inflammation occurring in adipose tissue of obese individuals is linked to the pathogenesis of insulin resistance. Although the exact trigger for this inflammatory process is unknown, adipose tissue hypoxia, ER stress, and SFA activation of innate immune processes have been identified as important processes in these disorders. In hypertrophic VAT, the induction of 12- and 5-LOX enzymes results in the increase in 12-HETE that has been linked to insulin resistance in adipocytes, and 5-HETE and production of chemotactic LTB_4 that may be an initiating factor in hypertrophic adipocytes to attract Mac2 macrophages and formation CLS structures (Chakrabarti et al., 2011). The increase in proinflammatory eicosanoids, 12-HETE and 5-HETE, results in induction of NF-κβ and secretion of pro-inflammatory insulin-resistant adipokines, macrophage inflammatory protein MIP-IΥ, TNFα, and IL-6 (Martinez-Clemente et al., 2011). The increased production of proinflammatory adipokines that cause insulin resistance can be reversed by the novel antiinflammatory drug lisofylline, which reduced p-STAT4 in VAT of obese Zucker rats and inhibited the inflammatory response, induced by LO products. These obese Zucker rats show a reduction in fasting plasma glucose and increase in insulin sensitivity (Chakrabarti et al., 2011). How inflammation-driven lipolysis in adipose tissue contributes to insulin resistance remains to be clearly established. TNFα clearly increases lipolysis in

adipocytes by both suppression of ATGL inhibitor GOS2 and PKA phosphorylation of ATGL activator, CGI-58/Abhd5 (Glass & Olefsky, 2012) as well as activation of HSL. The ability of salicylates to block the lipolytic response of TNFα (Zu et al., 2008) and the recent observation that LTB_4 receptor (BLT1)-deficient mice show reduction in Mac2 macrophage in VAT and reduction of proinflammatory cytokines secretion (Spite et al., 2011) suggest that activation of eicosanoid pathways are pivotal in adipose tissue inflammatory response and thus blockage of selective pathway might have insulin-sensitizing effects in obesity and MetS.

5.3. Eicosansoids in Adipocyte Metabolism and Obesity

Prostanoids have a significant role in adipose tissue mass remodeling where PGE_2 accelerates adipocyte accumulation of TAG through suppression of cAMP induction of HSL lipolysis. The prostanoid 15-deoxyPGI_2, a ligand for PPARγ activation, induces lipogenesis and ameliorates hyperglycemia in T2DM patients (Mazid et al., 2006). Prostacyclin and 15-deoxyPGI_2 also induce differentiation of preadipocytes, while $PGF_2\alpha$ inhibits preadipocyte differentiation. In the IVA PLA_2 knockout mice serum levels of PGE_2 are reduced, leading to reduced adipose mass and smaller adipocytes. Also, COX2-null mice have a significant reduction in body weight and percentage body fat due to increased metabolic rate. In the adipose tissue of COX2-null mice, the levels of 15-deoxyPGJ_2 and markers of adipocyte differentiation were significantly reduced, while preadipocyte marker expression increased along with a reduction in ATMs. Furthermore, the serum lipid lowering effects of niacin are due to promotion of adipogenesis through reduction of antiadipogenic $PGF_2\alpha$ and C/EBP activation of COX2, leading to possible synthesis of PGE_2 (Song et al., 2012). Adipocytes secrete a variety of adipokines that function to control whole-body metabolism: leptin, adioponectin, resistin, lipocalin2, and retinol binding protein 4 (RBP4) (Ouchi et al., 2011). The function of these adipokines is diverse in controlling the storage or use of lipid stores with leptin controlling nervous system appetite, resistin promoting insulin resistance through cytokine expression with lipocalin2 and RBP4, and adiponection having antiinflammatory and insulin sensitization properties. Adiponectin protects against hepatic steatosis by activation of AMPK and FA β-oxidation. Adiponectin production in adipocytes is inhibited by PGD_2, PGJ_2, and 15-deoxy PGI_2. Leptin expression is also inhibited by PGD_2, PGJ_2, and 15-deoxy PGI_2, but stimulated by PGE_2. Increased serum levels of resistin are related to the severity of NAFLD in humans, but no change in resistin levels were

noted in IVA PLA_2-null mice, indicating a minimal role of eicosanoids in controlling resistin production in adipocytes. These studies strongly indicate that PGs differentially regulate adipokine levels in MetS.

The key factors involved in HF-diet-induced adipose tissue inflammation and macrophage infiltration are not well understood. A recent study demonstrated that the 12/15-LOX pathway is upregulated in the adipose tissue of mice fed an HF diet and that 12/15-LOX metabolites induced adipose tissue inflammation and insulin resistance. This same observation of increased cytokine production and insulin resistance was observed in 3T3-L1 adipocytes incubated with FAs. In 12/15-LOX-null mice fed an HF diet, insulin resistance, proinflammatory M1 macrophage infiltration, and cytokine production by adipose tissue is dramatically reduced. In the adipose tissue of 15-LOX-null mice, there was a reduced expression of proinflammatory adipokines, MCP-1, TNFα and reduced resistin production with an increased expression and activity of the glucose transporter 4 (Glut4) (Martinez-Clemente, Ferre, Titos, et al., 2010). Remodeling of subcutaneous and omental adipose tissue occurs in obesity, MetS, and Cushing syndrome. In obese individuals, 15b-LOX and 12-LOX are expressed in both subcutaneous and omental fat, but only 15a-LOX is expressed in $CD34^+$ cells of subcutaneous fat where increased expression in associated with Homeostatis Model assessment-insulin resistance (HOMA-IR) (Dobrian et al., 2010). In obesity, FAs released from adipose stores are an important source of FAs that are found as TAG within hepatic LD. The regulation of lipid hydrolysis and synthesis of LDs is strongly associated with the differential regulation of LD proteins, perilipin, Cide/FSP27, and Serpin (Greenberg et al., 2011). Unfortunately, there are a limited number of studies that have investigated the role of LD proteins in NAFLD and whether eicosanoids regulate LD protein gene expression or protein function. In addition, the role of CYP2 epoxygenase and CYP4 ω-hydroxylase have not been studied in adipocytes and may be of importance considering the recent identification of FA ω-1-ethanol-inducible CYP2E1 expression in adipose tissue (Sebastian et al., 2011).

5.4. Eicosanoids in Diabetes and Insulin Resistance in Pancreas

The interplay of different organ systems, pancreas, adipose tissue, liver, skeletal muscle and nervous systems determine systemic insulin resistance in T2DM by signaling through different pathways that determine local organ-specific insulin resistance. FFA levels elevated in obese patients are an independent predictor of T2DM and coronary artery disease. Saturated FAs are

directly linked to pancreatic β-cell dysfunction through activation of TLR4/MyD88 pathway and induction of cytokines that recruit M1 monocytes/macrophages to pancreatic islets (Eguchi et al., 2012). This data firmly establishes a link between FA-induced islet inflammation and β-cell dysfunction in T2DM that is similar to the role of inflammation in adipose tissue. However, the functions of eicosanoids in different organ systems can have completely divergent effects in the regulation of metabolism. PGE_2 appears to be a significant factor in β-cell dysfunction and destruction in complications and pathogenesis of diabetes, while in adipose tissue, PGE_2 promotes adipogenesis, reduces serum FAs and glucose levels and thus protects against systemic insulin resistance of T2DM. In pancreatic islets, COX2-produced PGE_2 inhibits insulin secretion through autocrine activation of EP3 receptor, leading to decreased cAMP levels and response to GSIS. In contrast, PGI_2 increases cAMP through activation of IP receptor and potentiates GSIS. In human islets, COX2 is induced by high glucose (25 mM) and glycation products through advanced glycation end products, while it induces NF-κβ and IL-6 expression and COX promoter binding to induce COX2 gene expression. The inhibition of NF-κβ by sodium salicylates protects islet cells through reduced synthesis of COX2 and EP3 thereby preventing β-cell lost in diabetes. In addition, in liver, the protective effects of NSAID improve insulin resistance through PGE_2 involvement in the stimulation of glycogenolysis and gluconeogenesis (Luo & Wang, 2011).

AA is a potent stimulator of insulin secretion that is inhibited by 12/15-LOX activity and 12/15-LOX metabolites mediate cytokine damage to β-cells. In the nonobese diabetic (NOD) mouse model, mice develop autoimmune diabetes, but congenic NOD/12/15-LOX mice are protected from autoimmune diabetes (McDuffie et al., 2008). In 12/15-LOX-null mice fed an HF diet, islet damage was not observed. The functions of LOX metabolites in β-cell function are divergent with 15-HETE, LTB_4, and LTC_4 inhibiting insulin release and 12-HPETE potentiating insulin secretion (Dobrian et al., 2010). In diabetic Zucker rats, inhibition of 12-LOX activity suppressed AA-induced insulin secretion, while in 12-LOX-null mice, cytokines stimulated GSIS, indicating that 12-LOX products are negative regulators of insulin secretion (Zafiriou et al., 2011). There are three types of 12-LOX: platelet, leukocyte, and epidermal, with leukocyte 12-LOX being the major isoform expressed in pancreatic islets (Chen, Yang, Smith, Carter, & Nadler, 2005). 12-LOX expression is increased in islets of Zucker rats and the pancreatectomy model of diabetes, while 12-LOX expression in human islets by cytokines increases production of 12(S)-HETE that causes

β-cell apoptosis. LOX inhibitors protect islet cells from β-cell destruction in diabetic mice.

5, 6-EET produced by CYP epoxygenase stimulates insulin secretion in islets and is regulated by expression of CYP2CJ or inhibition of soluble epoxide hydratase (sEH) that metabolizes EETs to DHET. Inhibition of sEH promotes insulin secretion and GSIS in islets, and EETs have an antiapoptotic effect in β-cells. In sEH-null mice treated with streptozocin (STZ) to induce T1DM islet, cell apoptosis was significantly reduced compared to STZ-exposed wild-type mice, supporting the role EETs have in protecting β-cells from apoptosis. In both diabetes and obesity, the pancreatic expression of CYP2C isoforms is reduced (Zhao et al., 2005) with increased expression of CYP4A and sEH (Chen, Wang, et al., 2011). Of importance was the observation that insulin inhibits increased expression of CYP4A in diabetic rats. There are a number of studies associating sEH polymorphisms with insulin resistance in T2DM; however, there seems to be no association of variant alleles of CYP2C9, CYP2C8 or CYP2CJ2 in humans with either T1DM or T2DM (Surendiran et al., 2011). Further studies are necessary to determine the role of CYP2C and CYP4A AA metabolites in the function of β-cells and exocrine islet function in GSIS in hyperglycemia. Understanding how prostanoids regulate the function, proliferation and survival from cytokine- or high-FFA-mediated β-cell dysfunction will increase our understanding of the divergent effects of COX inhibitors on GSIS. Insight into how eicosanoid metabolism is regulated in the early development of T2DM characterized by hyperinsulin secretion to maintain normal glycemia followed by β-cell apoptosis may provide new avenues to protect β-cells from hyperglycemia.

5.5. Eicosanoids in Vascular and Cardiometabolic Diseases

Metabolic conditions such as obesity and insulin resistance lead to cardiovascular abnormalities caused by perturbation in lipid and carbohydrate metabolism. The functional role of eicosanoid pathways of PG, LT, and cytochrome P450 eicosanoids in vascular and cardiometabolic diseases has largely been studied in myocytes, endothelial and immune cells in ischemia, hypoxia, myocardial infarction, stroke, and hypertension. Unfortunately, the role of eicosanoids in the control of carbohydrate and lipid metabolism in these CVDs has not been extensively studied. The effective management of these diseases by NSAID provides therapeutic opportunities to selectively target eicosanoid pathways and prevent the underlying metabolic alteration of these diseases and ameliorate disease symptoms.

The risk of cardiovascular events during treatment with NSAIDs has been one of the most studied ADRs, especially with respect to disruption of vascular TXA2-PGI_2 balance by COX2 inhibitors. Similar to Coxib drugs, patients on diclofenac show a higher cardiovascular risk most likely due to metabolism and inactivation of diclofenac by CYP2C8, resulting in reduced synthesis of vasoprotective EETs in combination with the synthesis of pro-inflammatory eicosanoids (Fig. 5.2). It is imperative that we understand not only the mechanism of COX1 and COX2 channeling of PGH1 and PGH2 to selective synthase (isomerase) in the production of prostanoids but also the underlying metabolic changes in the target tissues. The differential expression and regulation of COX1 and COX2 by saturated and PUFAs make these enzyme amenable targets for not only drugs but also dietary manipulation by selective lipids. This is exemplified by palmitic acid stimulation of COX2 and inhibition of COX1 (Yuan, Sidhu, et al., 2009), and the preferential metabolism of AA over the antiinflammatory PUFA, EPA, by COX2. These results suggest that SFAs induce COX2 channeling of PGH_2 to different synthase that produce proinflammatory prostanoids. In contrast, EPA is a poor substrate for COX1 and at similar concentrations inhibits AA oxygenation.

The reader is referred to previous section on the role of phospholipase A_2 enzymes in CVD. The role of different prostanglandin synthases in vascular disease is revealed by their different roles in either the protection or aggravation of CVD in combination with their cognate GPCRs. Serum lipocalin PGD synthase is a marker of both coronary artery disease and atherosclerosis. Whether the effects of increased L-PGDS on coronary artery disease are due to increased synthesis of PGD_2 or the function of L-PGDS in transport of lipophilic lipids have not been determined. It is likely that detrimental effects of PGDS are not due to increased PGD_2 production since PGD_2 is metabolized by the AKR PGF synthase to antiinflammatory PGJ_2 that activates PPARγ (Smith et al., 2011). The inducible mPGES-1 is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism that have glutathione-transferase-mediated drug conjugation capabilities. mPGES-1 is associated with atherosclerosis since mPGES-1-null mice are protected from atherosclerosis but not hypertension through increased production of PGD_2. L-PGDS-null mice have atherosclerosis and obesity but no hypertension (Tanaka et al., 2009). PGF synthase of the AKR family metabolizes a number of substrates that include monosaccharides, steroids, xenobiotics, and prostanoids in the presence of NADPH. In vivo PGDS activity is attributed to AKR1B1 that is functionally coupled

to COX2 in $PGF_2\alpha$ synthesis in the vascular and arterial smooth muscle. Prostacyclin synthase (PGID-CYP8A1) produces PGI_2 that has a protective role in CVD and also functions as a PPARβ agonist. Like PGIS, TXAS is a cytochrome P450 (CYP5) that not only produces vasoconstrictive TXA_2 but also produces proinflammatory MDS and 12-HETE. TXAS is linked to COX2 in the PGH2 as a TXAS substrate.

A large number of studies clearly show LT involvement in several stages and types of CVD. Increased production of LTB_4 in human plaques and increased urinary LTE_4 production in patients with myocardial ischemia reveal that increase in 5-LOX activity correlated with the clinical stage of CVD. Both 5-LOX and BLT1 antagonists have protective effects on both ApoE-null and LDLR-null mice against atherosclerosis, myocardial infarction, and stroke. Increased 5-LOX in human artherosclerotic coronary arteries promote macrophage recruitment and production of vasoconstrictive LTC_4. In addition, BLT1-null mice show a variable artheroprotective effect by diminishing early but not late stages of lesion formation during intermittent hypoxia-induced atherogenesis (Li et al., 2011). Human Alox5 flap and LTA_4 hydrolase variants, which lead to increased LTB_4 synthesis, are associated with increased risk of myocardial infarction due to activation of endothelial and VSMC BLT1 (Helgadottir et al., 2006). In aortic angiotensin-II-induced aneurysm in ApoE-null mice, the intraluminal thrombus has increased expression of 5-LOX, FLAP and LTC_4 synthase that increases expression of MMP-2 in matrix degradation. The role of 12/15-LOX in atherosclerosis is controversial with overexpression of 15-LOX in macrophages protecting against atherosclerosis, while congenic 12/15-LOX/APOE-null mice are protected against atherosclerosis by production of proresolving lipids (Mesquita-Santos et al., 2011). Expression of both 5-LOX and 15-LOX2 was detected in human atherosclerotic plaques, but no 15-LOX1 enzyme can be detected, suggesting that 15-LOX promotes LDL oxidation and macrophage conversion to foam cells (Nakamura & Shimizu, 2011).

The eicosanoids of the ω-hydroxylase CYP4 pathway production of 20-HETE and epoxygenase CYP2 EET have largely opposing roles in cardiovascular inflammation with 20-HETE being proinflammatory and EETs being antiinflammatory; however, both eicosanoids are potent initiators of cell proliferation in the vascular system (Imig, 2012; Imig et al., 2001). CYP2J2 is highly expressed in the heart and produces EETs that attenuate I/R injury. The CYP cardioprotective effects of EETs are associated with activation of sarcolemmal and mitochondrial K^+ channels, MAPK,

and PI3K-AKT protective signaling pathways in endothelial and cardiac myocytes. It has also been shown that besides the generation of EETs by CYP2J2 and CYPC8, these P450 are uncoupled during EET formation and produce ROS that can compromise mitochondrial function, suggesting that cardiac CYP enzymes involved in NSAID metabolism can lead to drug efficacy or cardiotoxicity.

Within the past few years, the effects of lipid intermediary metabolism have been studied in regard to the risk of CVD in MetS. A relationship has been found between FADS activity with cardiometabolic risk factors of obesity, atherogenic lipoprotein phenotype and inflammation (Do et al., 2011). FADS activity is estimated indirectly by the product-precursor FA ratio of serum PL and cholesterol esters that are affected by both dietary fat intake and endogenous FA metabolism. It was found that a strong correlation exists between Δ6 FADS2 activity, waist circumference, and serum C-reactive protein in the risk for CVD. The desaturases affect lipid metabolism by both dietary intake and endogenous synthesis through Δ5FASD1 and Δ6FASD2 desaturation of dietary LA and linolenic acid. In contrast, Δ9 SCD1 is for the synthesis of palmitoelic and oleic acids for VLDL production. SCD1 in cardiomyocytes has a protective effect through inhibition of apoptosis, ceramide, DAG production, and generation of ROS (Matsui et al., 2012). In contrast, FASD1 Δ6-desaturase activity was positively associated with serum ICAM-1 and proinflammatory cytokines and negatively associated with adiponectin, suggesting that Δ6-desaturase synthesis of eicosanoids or PLs have a functionally undetermined role in cardiometabolic syndrome.

Eicosanoids recently have been shown to have a direct role in mitochondrial function in cardiometabolic diseases through dietary-induced increase in PL AA content in membrane PL. Replacement of mitochondrial PL with ω3-FAs prevented Ca^{2+}-induced mitochondrial depolarization and cell death through opening of the mitochondrial permeability transition pore (Moon et al., 2012). Furthermore, activation of mitochondrial phospholipase A2γ (PLA2γ-PNPLA8) increased synthesis of AA eicosanoids and inhibited long-chain acyl-CoA that modulates generation of eicosanoids that regulate mitochondrial bioenergetics and signaling functions. The roles of AA metabolites have a function in cell survival where LOX and COX eicosanoids induce NADPH oxidase and ROS formation (Cho et al., 2011). It has been shown that CYP4 20-HETE induces cardiomyocyte apoptosis through stimulation of caspase 3 activities and Bax expression (Bao et al., 2011). The relationship between acyl-CoA and eicosanoid function in

the mitochondria was revealed in aggressive breast cancer where ACSL4 increases intramitochondrial AA levels by ACSL4 induction of COX2 and generation of both LOX and COX eicosanoids that promote cell survival. It is not known how eicosanoids and FATP regulate mitochondrial function of cardiac myocytes in cardiometabolic diseases. There is a need to develop tissue-specific knockout of COX, LOX and cytochrome P450 eicosanoid enzymes in endothelial, VSMC, macrophages, and cardiomyocytes to identify the role of eicosanoids in cardiometabolic diseases and design effective eicosanoid-pathway-targeted treatments to prevent CVD.

6. THERAPIES IN THE TREATMENT OF NAFLD

NAFLD is one of the most common causes of chronic liver disease in adults and children worldwide. Even though simple steatosis is initially a benign condition, up to 5% of individuals with NAFLD can progress to chronic diseases of steatohepatitis (NASH), liver fibrosis, liver cirrhosis, and finally either end-stage liver disease or hepatocellular carcinoma. Therefore, early intervention is the key to limit disease progression and realize better outcomes. The cornerstone in the management of NAFLD should be exercise and efforts at weight reduction through dietary changes and increased physical activity. Unfortunately, the constellation of MetS diseases associated with NAFLD of insulin resistance, hypertriglyceridemia, hyperlipidemia, and hypertension often require the clinician to intervene and prescribe therapeutic modalities to control or reduce the progression of symptoms and diseases associated with MetS.

A number of clinical trials (http:www.ClinicalTrials.gov) for NAFLD and NASH have focused on the associated condition of MetS and to date no particular treatment has emerged as safe and effective. Pharmaceutical therapies have had mixed results and presently none have been accepted as a standard therapy. Most of the 78 clinical trials have focused on treating insulin resistance, hypertriglyceridemia, and hyperlipidemia. However, newer studies have begun to focus on the underlying metabolic alteration, oxidative stress, inflammation, and liver injury in the progression of NAFLD to NASH and ensuing chronic liver diseases. The majority of clinical trials for insulin resistance focus on incretin mimics (exenatide and Vildagliptin) to stimulate insulin levels or reduce serum glucose (acarbose-α-glucosidase inhibitor) or the AMPK activator, metformin. To treat hyperlipidemia, the standard has been to use 3-hydroxy-3-methylglutaryl-CoA statin inhibitors (atovastatin) and TZDs (pioglitazone) PPARγ agonist that decrease serum

glucose, lipid, inhibits inflammation and delays liver fibrosis. It is rather surprising that there are no trials for a combination study of statin and TZDs in the treatment of NAFLD or trials that use fibrates to increase FA β-oxidation. A rather new study uses arachidylamidocholanoic acid (Aramchol) that inhibits SCD-1 and upregulates ABCD1 in cholesterol transport. It is uncertain whether inhibition of SCD-1, which decreases steatosis, will increase liver injury as shown in SCD-1 knockout mice. Several studies are focused on treating the metabolic dysfunction by the use of lipoic acid, carnitine, and coenzyme Q targeted to the mitochondria (mitQ). In addition, a number of trials target the underlying causes of MetS, oxidative stress and inflammation by the use of vitamin E (Lovaza) (ω-3 fatty acids) and cysteamine to increase cysteine levels and therefore glutathione. In one randomized control trial, pentoxifylline, which suppresses TNFα synthesis and savages hydroxyl and peroxyl radicals, improves the histological features of NASH. Also, several trials have focused on the prevention of hepatic injury by the use of silymarin and the FXRα agonist 6-ethyl chenodoxycholic acid (obeticholic), which also has antiinflammatory properties.

It is rather disappointing that of the 38 NASH and 40 NAFLD clinical trials none have proposed direct the eicosanoid pathway by the use of salicylates and EPA/DHA for the treatment of NAFLD since salicylates activate AMPK and inhibit TNFα-induced adipose lipolysis, while combination with ω-3 FA produce potent antiinflammatory resolvins, protectins, and LX.

7. CONCLUSION

Although, COX, LOX, and P450 eicosanoid mediators elicit their own tissue- and cell-selective biological response, the cross-talk between synergistic and antagonistic lipid mediators within these pathways needs to be better understood in the disease process of MetS to design effective treatment with minimal ADRs. The goal will necessitate understanding the functional role of eicosanoids in the control of tissue-specific intermediary metabolism and in particular lipid and carbohydrate metabolism in the fundamental process of cell survival, apoptosis, and proliferation in NAFLD and MetS. Understanding how eicosanoids regulate metabolism is of particular importance considering the link between fatty transporters, channeling, and the differential regulation of eicosanoid pathways.

This challenge of defining tissue-specific eicosanoid pathway cross-talk in inflammation and metabolic disease can only be met by a bioinformatics

analysis of the transcriptome, proteome, lipidome, and metabolome in tissues affected by metabolic disease and inflammation in concert with analysis of tissue-specific eicosanoid flox knockout mice to design effective treatments. This fully integrated genomic, proteomic, and metabolomics analysis of eicosanoid pathways has recently been used to quantify eicosanoid metabolite production in macrophages in response to TLR4 signaling (Sabido et al., 2012) and thus adds invaluable insights into the integrated omics analysis of eicosanoid systems biology (Buczynski et al., 2009) http://www.lipidmaps.org. Extending this approach to drugs targeted to selective eicosanoid pathways with analysis of drug metabolism pathways (Guengerich & Cheng, 2011) provides a novel approach to design effective tissue-specific targeted therapies with the reduced ADRs and greater efficacy in the treatment of inflammatory and diseases of MetS and NAFLD (http://bioinformatics.charite.de/supertarget/).

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ABBREVIATIONS

AA Arachidonic acid
ABC ATP-binding cassette transporter
ACBP Acyl-CoA binding protein
ACS Acyl-CoA synthetase
ACSL Long-chain acyl-CoA synthetase
ACSVL Very-long-chain acyl-CoA synthetase
ACOX Acyl-CoA oxidase
AGPAT Acyl-CoA:1-acylglycerol-3-phosphate acyltransferase (LPAAT)
ALA alpha-linoleic acid
AMPK AMP protein kinase
AP1 Activator protein
APOE apoE lipoprotein
ATGL Adipose triglyceride lipase
ATM Adipose tissue macrophage
BLT LTB4 receptor
CD36/FAT Fatty acid translocase
COX Cylooxygenase
CL Cardiolipin
cysLT LTC4,LTD4,LTE4 receptor

CYP Cytochrome P450
CVD Cardiovascular disease
DAG Diacylglycerol
DAGT Diacylglycerol transferase
DHA Docosahexaenoic acid
DIO Diet-induced obesity
DP Receptor for PGD
EDP epoxydocosapentaenoic acid
EED DHA CYP2-produced epoxydocosapentaenoic acid
EEQ EPA CYP2-produced epoxyeicosatetraenoic acid
EET Epoxyeicosatrienoic acid
EHHADH Enoyl-CoA hydratase 3-hydroxyacyl-CoA dehydrogenase
ELOVL Elongase enzyme
EP Receptor for PGE
EPA Eicosapentaenoic acid
ERKS extracellular receptor kinase 2
Exoins Proinflammatory 15-LOX eicosanoids
FABP Fatty-acid-binding protein
FADS Fatty acid desaturase
FATP Fatty acid transport proteins
FFA Free fatty acid
FLAP 5-lipoxygenase activity protein
FP Receptor for PGF
FXR Farnesoid-X-receptor
Gα (q/11) G α subunit q that activates phospholipase c
GEF guanine exchange factor
GPAT Glycerol-3-phosphate acyltransferase
GSH Reduced glutathione
GST Glutathione-S-transferase
HDL High-density lipoprotein
HEET hydroxyepoxyeicosatrienoic acid
HETE Hydroxeicosatetraenoic acid
HNF4 Hepatocyte nuclear factor 4
HODE Hydroxyoctadecadienoic acid
HSL Hormone-sensitive lipase
HSPG Heparin sulfate proteoglycan
IL Interleukins
IP Receptor for PGI
IRS Insulin receptor substrate
LD lipid droplets.
LDLR Low-density lipoprotein receptor
Lipin Phosphatidic acid phosphatase
Lipoxin Antiinflammatory LTA4
LOX Lipoxygenase
LTB4 Leukotriene B4
LPA Lysophosphatidic acid
LPAAT lysophosphatic acid acyltransferase

LPCAT Lysophosphatidylcholine acyl-CoA transferase
LPLAT lysophospholipid acid acyltransferase
LysoPC Lysophosphatidylcholine
LXR Liver-X-receptor
MAPK2 mitogen activated protein kinase 2
MBOAT Membrane-bound O-acyltransferase
MCP-1 monocyte chemotactic protein
MGAT Monoglycerol acyltransferase
MGST microsomal GSH transferase
MetS Metabolic syndrome
MRP Multidrug resistant protein
NAFLD Nonalcoholic fatty liver disease
NASH Nonalcoholic steatohepatitis
NF-κβ Nuclear factor kappa β
NSAID Nonsteroidal antiinflammatory drug
Null Gene knockout
OAT Organic anion transporter
Omega-3 PUFA ω3-PUFA
PAP Phosphatidic acid phosphatase
PNPLA Patatin-like phospholipase domain containing lipases
PGC Peroxisome proliferator activated receptor coactivator
PLA2 Phospholipase A2
PA Phosphatidic acid
PC Phosphatidylcholine
PE Phosphatidylethanolamine
PG Prostaglandin
PGD_2 prostaglandin D_2
PGDS PGD synthase
PGE_2 prostaglandin E_2
$PGF_{2\alpha}$ prostaglandin E_2
PGES PGE synthase
PGFS PGF synthase
PGHS Prostaglandin endoperoxide H synthase
PGG2 Protaglandin peroxidase
PGH_2 prostaglandin endoperoxide
PGI Prostacyclin
PGIS Prostacyclin synthase
PGJ_2 prostaglandin J_2
PKA Protein kinase A
PKC Protein kinase C
PL Phospholipid
PLIN perilipin
PS Phosphatidylserine
PPAR Peroxisome proliferator activated receptor
PUFA Polyunsaturated fatty acid
PXR Pregnane-X-receptor
RAR Retinoic acid receptor
RXR Retinoid-X-receptor

SCD-1 Stearoyl-CoA desaturase
sEH Soluble epoxide hydrolase
SLC Solute ligand carrier
SFA Saturated fatty acid
SRSA Slow-reacting substance of anaphylaxis
SREBP Sterol regulatory-element-binding protein
TAG Triacylglycerol
T1DM Type I diabetes mellitus
T2DM Type II diabetes mellitus
TX Thromboxane
TXAS Thromboxane synthase
TXA_2 thromboxane A2
TZD Thiazolidinedione
usFA Unsaturated fatty acid
VLDL Very-low-density lipoprotein
20-HETE AA CYP4-produced hydroxyeicosatetraenoic acid
19-HEPE EPA CYP4-produced hydroxyeicosapentaenoic acid
22-HDoHE-DHA CYP4 Hydroxdocosahexaenoic acid

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CHAPTER SIX

The Yin and Yang of Protein Kinase C-theta (PKCθ): A Novel Drug Target for Selective Immunosuppression

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Contents

Abstract

Protein kinase C-theta (PKCθ) is a protein kinase C (PKC) family member expressed predominantly in T lymphocytes, and extensive studies addressing its function have been conducted. PKCθ is the only T cell-expressed PKC that localizes selectively to the center of the immunological synapse (IS) following conventional T cell antigen stimulation, and this unique localization is essential for PKCθ-mediated downstream signaling. While playing a minor role in T cell development, early in vitro studies relying, among others, on the use of PKCθ-deficient (*Prkcq*$^{-/-}$) T cells revealed that PKCθ is required for the activation and proliferation of mature T cells, reflecting its importance in activating the transcription factors nuclear factor kappa B, activator protein-1, and nuclear factor of activated T cells, as well as for the survival of activated T cells. Upon subsequent analysis of in vivo immune responses in *Prkcq*$^{-/-}$ mice, it became clear that PKCθ has a selective role in the immune system: it is required for experimental Th2- and Th17-mediated

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allergic and autoimmune diseases, respectively, and for alloimmune responses, but is dispensable for protective responses against pathogens and for graft-*versus*-leukemia responses. Surprisingly, PKCθ was recently found to be excluded from the IS of regulatory T cells and to negatively regulate their suppressive function. These attributes of PKCθ make it an attractive target for catalytic or allosteric inhibitors that are expected to selectively suppress harmful inflammatory and alloimmune responses without interfering with beneficial immunity to infections. Early progress in developing such drugs is being made, but additional studies on the role of PKCθ in the human immune system are urgently needed.

1. INTRODUCTION

The immune system is an immensely complex array of different cell types and soluble products—antibodies, cytokines, chemokines, growth factors and other mediators—that have evolved in order to protect us against the many pathogens that we face throughout our life. Because we face a multitude of danger signals presented by bacteria, viruses, fungi, parasites and growing tumors that can potentially harm us by acting on different cell types and tissues in the body via a large number of distinct mechanisms of action, the immune system has to be equally diverse and multifaceted in order to effectively protect us against diseases and ensure our health and survival. Thus, many layers of regulation exist in the immune system to maximize the probability that immune responses are sufficient to afford protection, but are not excessive so as to provoke harmful tissue inflammation. Thus, for almost every action of the immune system, there is a reaction. A prime example of this sophisticated regulation is represented by regulatory T cells (Tregs), which function to maintain immune homeostasis and dampen excessive immune responses (Josefowicz et al., 2012; Rudensky, 2011; Sakaguchi et al., 2008). However, this regulatory complexity of the immune system comes at a potentially heavy price, namely, when genetic predisposition or environmental factors disturb and alter the fine balance between beneficial and harmful immunity, the outcome, in the form of inflammation and autoimmunity, can result in debilitating, and sometimes fatal diseases. For example, humans and mice lacking functional Tregs due to mutations in the *Foxp3* gene succumb to a severe lymphoproliferative and inflammatory disease (Bennett et al., 2001; Brunkow et al., 2001; Gambineri et al., 2003). Hence, a major goal of immunology research has been to understand the regulatory mechanisms that operate in the immune system, with the ultimate goal of developing therapeutic strategies for diseases and conditions that result from altered and/or undesired immune responses,

be it therapies designed to dampen undesired immune responses, such as autoimmune diseases, inflammation and transplant rejection, or immune interventions aimed at boosting desired responses such as antitumor immunity or viral clearance in immunosuppressed individuals (e.g. HIV infection and AIDS).

Given the critical role of T lymphocytes in controlling and mediating various types of immune responses, it is not surprising that T cells have served, and continue to serve, as logical and major drug targets for treating immunological diseases and cancer. Various treatment modalities that consist of T cell depletion, alteration of T cell adhesion and trafficking, potentiation or inhibition of costimulatory receptors, modulation of cytokines and their signaling pathways, and intervention in T cell receptor (TCR) signaling pathways have been devised and applied clinically with different degrees of success (Steward-Tharp et al., 2010). However, most of these drugs and treatments are not sufficiently specific and, as a result, have undesirable toxic side effects. This is the case with the major component of immunosuppressive drug combinations, i.e. calcineurin (CN) inhibitors such as cyclosporine A (tacrolimus), or with the use of anti-CD3 antibodies to deplete T cells—treatments which prevent organ transplant rejection, graft-*versus*-host disease (GvHD) and other undesired immune responses but, at the same time, also render treated patients susceptible to infection due to their immunosuppressed status (Riminton et al., 2011). Hence, a major effort in recent years has gone toward the rational development of more effective immune therapies, which display increased selectivity and reduced toxicity. An emerging promising drug target that falls into this category is protein kinase C-theta (PKCθ), an enzyme that is predominantly expressed in T cells, where it plays critical roles in TCR signaling pathways. Of particular importance, recent evidence, mostly based on animal studies, indicates that the requirement for PKCθ is quite selective—deleterious immune responses such as autoimmunity strongly depend on it, while it is dispensable for other, beneficial forms of immunity such as protection against viral infections. Hence, there is currently substantial interest in targeting PKCθ as a means of selectively modulating immunity in favor of the patient. Several relatively recent articles have reviewed the history, functions and regulation of this enzyme (Hayashi & Altman, 2007; Isakov & Altman, 2012; Sun, 2012; Zanin-Zhorov et al., 2011). Therefore, this communication is not meant to provide a comprehensive review of everything that is known about PKCθ but, rather, serves as a compact summary of current knowledge about this enzyme and, in particular, highlights those

of its known functions in T cell biology that make it an increasingly attractive immunomodulatory drug target. In addition, we also define important open questions, and provide future perspectives related to PKCθ.

2. HISTORY, STRUCTURE AND EXPRESSION OF PKCθ

The protein kinase C (PKC) family consists of serine/threonine kinases that mediate a wide variety of cellular processes (Baier, 2003; Hug & Sarre, 1993; Mellor & Parker, 1998; Newton, 1997; Nishizuka, 1995). PKC, which was initially purified from brain extract, was defined as a novel cyclic nucleotide-independent, lipid- and Ca^{2+}-dependent enzyme (Inoue et al., 1977; Takai et al., 1979), and later found to serve as the cellular receptor for tumor-promoting phorbol esters (Castagna et al., 1982; Kikkawa et al., 1983; Niedel et al., 1983). Initially considered to be a single entity, it later became clear, with the molecular cloning of the cDNAs encoding the first three members of the PKC family, PKCα, β and γ (Coussens et al., 1986; Parker et al., 1986), soon to be followed by the isolation of additional related enzymes, that PKC constitutes a new family of enzymes, which now contains 10 defined members.

All PKCs are composed of an N-terminal regulatory domain and a C-terminal catalytic domain that are separated by a flexible hinge region, also known as the V3 domain. Based on structural differences in the regulatory domain and cofactor requirements for activation, PKCs are subdivided into three subfamilies: conventional (or classical) PKCs (cPKCs; α, βI, βII, and γ), novel PKCs (nPKCs; δ, ε, η, and θ) and atypical PKCs (aPKCs; ζ and ι/λ). The regulatory region of cPKCs contains three lipid-binding domains; two contiguous cysteine-rich C1 domains (C1A and C1B; ~50 residues each) involved in binding of the second messenger diacylglycerol (DAG) or phorbol esters, followed by an N-terminal C2 domain (~130 residues) that is responsible for Ca^{2+}-dependent binding to membrane-localized phosphatidylserine or other phospholipids. Hence, binding of both DAG and Ca^{2+} to the C2 and C1 domain, respectively, is required for the activation of cPKCs. The nPKCs also have two tandem DAG-binding C1 domains and an N-terminal C2 domain, which, however, does not bind Ca^{2+} but may bind phospholipids. DAG binding to the tandem C1 domains activates nPKC subfamily members. The positioning of the C2 and C1 domains in nPKCs is reversed relative to the cPKCs, such that the tandem C1 domains follow the N-terminal C2 domain. Finally, the aPKCs have only one C1 domain that does not

bind DAG, and the cofactors or mechanisms that activate aPKCs are less well understood. In addition, the regulatory region of all PKCs contains a short conserved pseudosubstrate sequence corresponding to an ideal PKC substrate recognition site, with the exception that a Ser/Thr residue, which would normally be phosphorylated, is replaced by an Ala residue (Baier, 2003; Pfeifhofer-Obermair et al., 2012).

The discovery of PKC enzymes as cellular receptors for phorbol esters provided a potential explanation for the T cell mitogenic and activating properties of phorbol esters (Abb et al., 1979; Touraine et al., 1977). A separate group of studies at about the same time documented the ability of a combination of phorbol esters and Ca^{2+} ionophores to mimic TCR and costimulatory signals (such as those provided by CD28, the prototypical T cell costimulatory receptor) leading to T cell activation and proliferation (Isakov & Altman, 1985; Kaibuchi et al., 1985; Truneh et al., 1985a, 1985b). Together, these two sets of independent findings implicated PKCs as potentially important players in T cell activation. Subsequently, three independent groups, including ours, cloned human and mouse cDNAs encoding a new member of the PKC family termed PKCθ (Baier et al., 1993; Chang et al., 1993; Osada et al., 1992). As it turned out later, the discovery of PKCθ was only the "opening shot" to an extensive series of studies by many groups, which have revealed (and continue to do so) the importance of this unique PKC family member in several cell types, but particularly in T lymphocytes.

Chromosomal mapping located the human PKCθ gene (*Prkcq*) to the short arm of chromosome 10 (10p15) (Erdel et al., 1995), a region prone to mutations that lead to T cell leukemias, lymphomas and T cell immunodeficiencies (Monaco et al., 1991; Verma et al., 1987). The *Prkcq* gene has an open reading frame corresponding to a protein with 706 amino acid residues having a molecular weight of ~79–81 kD, which consists of an amino-terminal regulatory domain (amino acids ~1–378) and a carboxy-terminal catalytic domain (amino acids ~379–706). The hinge/V3 domain, representing a part of the regulatory domain, consists of residues ~291–378 (Baier et al., 1993; Chang et al., 1993; Xu et al., 2004). The crystal structure of the PKCθ catalytic domain has been solved (Xu et al., 2004), revealing that PKCθ displays two main conformational states, i.e. an "open/active" and a "closed/inactive" state (Seco et al., 2012; Xu et al., 2004). The allosteric change of PKCθ from a "closed" to an "open" state involves two important mechanisms: DAG binding to the C1 domains and phosphorylation

of Thr-538 (T538) in the activation loop (Budde et al., 2010; Seco et al., 2012), which is most likely constitutively phosphorylated, resulting in a constitutively competent, but not fully active, kinase (Liu et al., 2002). The interface of the regulatory and catalytic domains constitutes the active site cleft, which is responsible for the substrate binding and phosphate delivery from the active catalytic site to the substrate. In addition to Thr-538, whose phosphorylation is essential for kinase activation, there are several other phosphorylation sites in PKCθ (Freeley et al., 2005; Liu et al., 2002; Liu et al., 2000; Thuille et al., 2005; Wang et al., 2012), some of which are shared by other PKCs (Ser-676 and -695 in PKCθ), and others being unique to PKCθ (Tyr-90 and Thr-219). These phosphorylation sites play distinct role in controlling the activity and/or cellular localization of PKCθ (Wang et al., 2012).

PKCθ is most abundant in hematopoietic cells, especially T cells (Baier et al., 1993). The high expression level of PKCθ in T cells accounts for the abundance of this enzyme in the thymus and lymph nodes, with lower levels in spleen, and undetectable expression in the bone marrow (Meller et al., 1998; Meller et al., 1999). In addition to T cells, PKCθ is readily detected in mast cells, natural killer (NK) cells and platelets, but not in B cells, erythrocytes, neutrophils, monocytes, or macrophages (Liu et al., 2001; Meller et al., 1998; Meller et al., 1999; Vyas et al., 2001). High expression level of PKCθ is also observed in skeletal muscle (Baier et al., 1993; Chang et al., 1993; Meller et al., 1998; Osada et al., 1992), where PKCθ has been implicated in mediating insulin resistance associated with type 2 diabetes (Griffin et al., 1999; Itani et al., 2000; Kim et al., 2004; Serra et al., 2003). Analysis of PKCθ mRNA expression during mouse development revealed expression in yolk sac blood islands and in the liver, and later in the thymus and skeletal muscle. In addition, high expression was detected in the embryonic nervous system, including spinal ganglia, spinal cord, trigeminal and facial ganglia and a subsection of the thalamus (Bauer et al., 2000).

3. SPECIALIZED FUNCTIONS OF PKCθ IN CONVENTIONAL T CELLS: THE YIN

Given the important role of PKCθ in TCR-mediated T cell activation, it is worthwhile to briefly review the major features of TCR signaling. TCR ligation by a peptide antigen–major histocompatibility complex (MHC)

complexes together with the engagement of CD28 by its ligand, B7, leads to the activation of Src-family tyrosine kinases (Lck and Fyn), which phosphorylate the immunoreceptor tyrosine-based activation motifs in the TCR-ζ chains and CD3 subunits (Chan & Shaw, 1996). Recruitment and activation of ZAP-70 and Tec-family tyrosine kinases follow, resulting in the phosphorylation and activation of additional enzymes and adapter proteins and formation of multicomponent signaling complexes that ultimately activate various downstream signaling pathways. These events culminate in the activation of key transcription factors (activator protein (AP)-1, nuclear factor kappa B (NF-κB) and nuclear factor of activated T cells (NFAT)), which induce gene expression programs leading to T cell activation and proliferation (Kane et al., 2000; Samelson, 2002). Two key early events in TCR signaling are the tyrosine phosphorylation-dependent activation of the linker for activation of T cells (LAT), which serves as a scaffold for the recruitment of signaling proteins and assembly of a TCR signalosome (Wange, 2000), and the activation of phospholipase Cγ1 (PLCγ1), which hydrolyzes membrane inositol phospholipids to generate two second messengers that activate two bifurcating signaling pathways: IP_3 initiates Ca^{2+} signaling pathways, and DAG activates PKC and some other targets, including Ras signaling (Kane et al., 2000; Samelson, 2002). Thus, the activation of cPKCs and nPKCs in T cells lies on the pathway initiated by DAG.

T cells express at various levels up to eight distinct members of the PKC family, i.e. PKCα, β, δ, ε, η, θ, ζ and ι (Baier, 2003; Hug & Sarre, 1993; Pfeifhofer-Obermair et al., 2012). The expression of multiple PKC isoforms in T cells suggests functional redundancy and possible specialization. Indeed, T cell-expressed PKCs other than PKCθ have been reported to control various functions in T cells (Baier, 2003; Pfeifhofer-Obermair et al., 2012). Nevertheless, the predominantly high expression of PKCθ in T cells has suggested that it might play potentially unique and nonredundant functions in T cell biology. The first clue pointing in this direction was a report that PKCθ, but not PKCα, activated the transcription factor AP-1, which is required for productive T cell activation. However, it was not until *Prkcq*$^{-/-}$ mice were generated that the tools required to directly address the question of redundancy became available (Pfeifhofer et al., 2003; Sun et al., 2000).

Prkcq$^{-/-}$ mice are generally healthy and fertile, and early studies indicated that T cell development was intact in these mice (Pfeifhofer et al., 2003; Sun et al., 2000). A later study using TCR-transgenic mice expressing a lower avidity TCR revealed a substantial, albeit not absolute, role for PKCθ in mediating positive selection in the thymus (Morley et al., 2008).

In this regard, PKCθ and another nPKC, PKCη, seem to behave in a partially redundant manner (Fu et al., 2011). The most prominent defect in *Prkcq*$^{-/-}$ mice was, however, the severely impaired TCR-mediated activation of mature, peripheral T cells. In both *Prkcq*$^{-/-}$ mouse models, impaired responses to TCR/CD28-induced stimulation were observed in proliferation, IL-2 production, NF-κB and AP-1 activation. The original finding of impaired NF-κB activation in *Prkcq*$^{-/-}$ T cells coincided with in vitro biochemical studies that similarly established NF-κB as being a major target of PKCθ, reflecting the PKCθ-dependent activation of IκB kinase-β (IKKβ), but not IKKα (Coudronniere et al., 2000; Lin et al., 2000). However, there were some notable differences between the two *Prkcq*$^{-/-}$ mouse models: Whereas *Prkcq*$^{-/-}$ T cells displayed impaired TCR/CD28-induced CD25 and CD69 upregulation and phorbol ester plus ionomycin-induced proliferation in one study (Sun et al., 2000), the same responses were normal in the other report (Pfeifhofer et al., 2003). Conversely, NFAT activation was found to be impaired (Pfeifhofer et al., 2003) or intact (Sun et al., 2000). Some of these differences may be related to the different strategies used by the two groups to generate *Prkcq*$^{-/-}$ mice. Thus, Littman et al. inactivated the *Prkcq* gene by homologous recombination in embryonic stem cells via replacement of the exon encoding the ATP-binding site of the kinase with a neomycin resistance gene (Sun et al., 2000), potentially resulting in residual expression of the N-terminal regulatory region. Baier et al. generated a null *Prkcq* allele by using the Cre/LoxP system to delete exons 3 and 4 encoding amino acid residues 10–87, resulted in a frame shift after amino acid residue 9 of mouse PKCθ and essentially, a complete deletion of the corresponding protein (Pfeifhofer et al., 2003). Nevertheless, later studies using *Prkcq*$^{-/-}$ mice generated by Littman et al. (Sun et al., 2000) demonstrated that, in fact, Ca^{2+} signaling and NFAT activation are impaired in T cells from these mice (Altman et al., 2004; Manicassamy et al., 2006), raising the possibility that the use of saturating, nonphysiological anti-CD3/CD28 antibody concentrations in the original study (Sun et al., 2000) likely masked the more subtle effects of *Prkcq* deletion on Ca^{2+} signaling. Hence, PKCθ regulates to various degrees all three transcription factors required for productive T cell activation, i.e. NF-κB, AP-1, and NFAT, accounting for the impaired proliferation and cytokine production by *Prkcq*$^{-/-}$ T cells.

Among the three transcription factors that are regulated by PKCθ and are known to be important for productive T cell activation, the pathway leading

from PKCθ to NF-κB activation has been analyzed most extensively. Several enzymes and adapter proteins play a role in TCR-mediated activation of the canonical NF-κB (NF-κB1) pathway. These include caspase recruitment domain (CARD), membrane-associated guanylate kinase protein-1 (CARMA1, also termed CARD11), B-cell lymphoma-10 (Bcl10), mucosa-associated lymphoid tissue-1 (MALT1), and the IKK complex (Lin & Wang, 2004; Weil & Israel, 2004). The latter consists of two enzymatic components, IKKα and IKKβ, and a regulatory subunit, IKKγ (also known as NEMO). CARMA1 is constitutively associated with lipid rafts, and becomes further enriched in these rafts after TCR stimulation. Following T cell activation, PKCθ phosphorylates CARMA1 on several serine residues, a modification essential for the ability of CARMA1 to activate NF-κB (D. Wang et al., 2002). PKCθ-phosphorylated CARMA1 recruits the Bcl10–MALT1 complex, which then activates IKK by inducing ubiquitination and degradation of IKKγ, allowing activated IKKβ (and perhaps IKKα) to phosphorylate the inhibitory IκB proteins. This, in turn, results in IκB degradation and, consequently, in NF-κB1 nuclear translocation and activation (Ghosh & Karin, 2002).

The pathway leading from PKCθ to AP-1 activation is less clearly understood. AP-1 activation depends on several mitogen-activated protein (MAP) kinases (Shaulian & Karin, 2002). Despite early findings that the TCR/CD28-induced activation of MAP kinases in *Prkcq*$^{-/-}$ T cells is intact (Pfeifhofer et al., 2003; Sun et al., 2000), we found more recently that *Prkcq*$^{-/-}$ CD8^{+} T cells displayed impaired extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activation in response to specific antigen stimulation (Barouch-Bentov et al., 2005). Thus, the importance of PKCθ in MAP kinase activation may have been masked by costimulation with saturating concentrations of anti-CD3/CD28 antibodies (Pfeifhofer et al., 2003; Sun et al., 2000). The PKCθ-mediated AP-1 activation is dependent on Ras (Baier-Bitterlich et al., 1996) and we have found that SPAK, an Ste20-related MAP kinase, is a direct interactor and substrate of PKCθ in the pathway leading to AP-1, but not NF-κB, activation (Li et al., 2004). Least understood of all is the mechanism that links PKCθ to the activation of NFAT, but it may involve Tec-family tyrosine kinases such as Itk and Tec as PKCθ interactors that link it to PLC-γ1 activation (Altman et al., 2004).

In addition to its importance in T cell activation, PKCθ has been shown to play an important role in the survival of T cells. This effect may involve several distinct mechanisms. The first mechanism is related to the process of

activation-induced cell death in T cells, whereby binding of FasL, the ligand for the major death receptor Fas (CD95) expressed on activated T cells, triggers the death of these cells via an extrinsic apoptotic pathway. Thus, PKCθ and CN cooperated to induce expression of FasL (Villalba et al., 1999; Villunger et al., 1999), and full activation of the *Fasl* gene promoter required binding sites for the three major transcription factors positively regulated by PKCθ, namely, AP-1, NF-κB and NFAT (Villalba et al., 1999), the latter being a prominent target of CN. Along the same line, the Fas-mediated lytic activity of cytotoxic T lymphocytes (CTLs) was also found to involve a PKCθ-dependent pathway of FasL upregulation (Pardo et al., 2003). Second, PKCθ (but also another nPKC, PKCε) were found to rescue T lymphocytes from Fas-mediated apoptosis via phosphorylation and inactivation of Bcl2-associated death promoter (BAD) (Bertolotto et al., 2000; Villalba et al., 2001), a Bcl2 family member that antagonizes the effect of the prosurvival proteins Bcl2 and Bcl_{xL}, by physically associating with them. Similarly, PKCθ was required for the survival of both activated $CD4^+$ (Manicassamy et al., 2006; Saibil, Jones, et al., 2007) and $CD8^+$ T cells (Barouch-Bentov et al., 2005; Saibil, Jones, et al., 2007) by regulating the expression of Bcl2 family proteins, i.e. increasing the expression of the antiapoptotic proteins mentioned above (Bcl2 and Bcl_{xL}) and, conversely, suppressing expression of the related proapoptotic protein Bim_{EL}. c-Rel, a component of the NF-κB1 transactivating complex, seems to link PKCθ to this survival signal (Saibil, Jones, et al., 2007).

In addition to its function as a signal transducer from the TCR and CD28 on the T cell surface, PKCθ most likely also has biologically relevant nuclear functions. The first clue for such a function came from a report that PKCθ, but not other tested PKCs, associates with centrosomes and kinetochore structures of the mitotic spindle within the nucleus of murine erythroleukemia cells, suggesting a role in cell proliferation (Passalacqua et al., 1999). More recently, it was found that PKCθ physically associates with the proximal promoter and coding regions of inducible immune response genes in human T cells (Sutcliffe et al., 2011). Chromatin-tethered PKCθ formed an active nuclear complex by associating with RNA polymerase II, the histone kinase MSK-1, and the adapter protein 14-3-3ζ. Furthermore, a chromatin immunoprecipitation (ChIP)-on-ChIP assay demonstrated that PKCθ also localizes to the regulatory regions of a distinct cluster of microRNA promoters and negatively regulates their transcription (Sutcliffe et al., 2011).

4. THE DIFFERENTIAL ROLE OF PKCθ IN IMMUNE RESPONSES

The severe defects observed early on in the in vitro activation, proliferation and IL-2 production by *Prkcq*$^{-/-}$ T cells (Pfeifhofer et al., 2003; Sun et al., 2000) generally led to the notion that PKCθ is globally required for all T cell-mediated immune responses, raising doubts about its utility as a drug target for immunosuppression. The concern was that similar to the widely used immunosuppressive drugs such as CN inhibitors (e.g. tacrolimus), inhibition of PKCθ would nonselectively suppress desired immune responses and render patients susceptible to infections. It was not until 2004, namely 4 years after the generation of the first *Prkcq*$^{-/-}$ mouse model (Sun et al., 2000), that the first report analyzing in vivo immune function of *Prkcq*$^{-/-}$ mice has appeared, documenting the somewhat surprising finding that CTL and antibody response against vesicular stomatitis virus (VSV) infection were intact in these mice (Berg-Brown et al., 2004). This study was soon followed by additional reports by many other groups, leading to the now widely accepted view that the requirement of PKCθ for immune responses is, in fact, quiet selective (Table 6.1), providing one of the strongest arguments for the promise of this enzyme as a useful drug target.

The first report that PKCθ is dispensable for antiviral responses mediated by CTLs was confirmed by several other groups in the context of several different viral or bacterial infection models (Giannoni et al., 2005; Marsland et al., 2005; Valenzuela et al., 2009), although one of these studies reported reduced antiviral antibody and type 1 helper T (Th1) responses (Giannoni et al., 2005). The relative importance of PKCθ in protective immunity against pathogen infection is likely determined in part by the pathogen load, as indicated by finding that *Prkcq*$^{-/-}$ mice can clear *Listeria monocytogenes* infection when inoculated with 2×10^3 colony-forming units of bacteria (Valenzuela et al., 2009), but not when a 25-fold higher bacterial load is used (Sakowicz-Burkiewicz et al., 2008). These findings suggest that alternative signals such as innate immunity provided by infection with live pathogens can compensate for the lack of PKCθ in vivo and allow an adequate protective response. Indeed, more recent studies demonstrated that increased activation signals delivered in vivo by highly activated dendritic cells (Marsland et al., 2005) or by a toll-like receptor (TLR) ligand (Marsland et al., 2007), as present during viral infections, overcome the requirement for PKCθ during CD8$^+$ T cell antiviral

Table 6.1 Selectivity of PKCθ functions in vivo

In vivo immune responses	Role of PKCθ	Immune cells implicated in response	References
Effector function/ memory responses/viral clearance upon LCMV infection	Dispensable	CTL, Th1	Berg-Brown et al. (2004)
Neutralizing antibodies against VSV infection	Dispensable	B cells, Tfh help	Berg-Brown et al. (2004)
Murine herpes virus-68 clearance, expansion of virus-specific $CD8^+$ T cells	Dispensable	CTL	Giannoni et al. (2005)
Recall responses to vaccinia virus infection	Dispensable	CTL	Marsland et al. (2005)
Murine CMV clearance, expansion of virus-specific $CD8^+$ T cells	Dispensable	CTL	Valenzuela et al. (2009)
Effector CTL response to *Listeria monocytogenes* (LM) infection★	Dispensable	CTL	Valenzuela et al. (2009)
LM clearance, effector cell expansion†	Required	CTL, Th1	Sakowicz-Burkiewicz et al. (2008)
Effector response against *T. gondii* infection, pathogen clearance	Required	Th1, CTL, Th2, B cells	Nishanth et al. (2010)
Plasmodium berghei ANKA-induced Inflammatory cerebral malaria	Moderately required	Th1, CTL?	Ohayon et al. (2010)
Leishmania major clearance, effector response against infection	Dispensable (B6) Required (Balb/C)	Th1 Th2	Marsland et al. (2004)
Immunity to M-MuLV-induced leukemia	Required	CTL, Th1	Garaude et al. (2008)
Rejection of engrafted MHC class I-negative tumors	Required	NK	Aguilo et al. (2009)
Lung inflammation induced by ovalbumin administration	Dispensable Required	Th1‡ Th2§	Salek-Ardakani et al. (2004), Marsland et al. (2004)

Table 6.1 Selectivity of PKCθ functions in vivo—cont'd

In vivo immune responses	Role of PKCθ	Immune cells implicated in response	References
IgE, eosinophilia response to *N. brasiliensis* infection	Required	Th2	Marsland et al. (2004)
GvL response	Dispensable	Th1, CTL?	Valenzuela et al. (2009)
Systemic GvHD	Required	Th1, CTL	Valenzuela et al. (2009)
Local (footpad) host *vs* graft response	Required	Th1, CTL	Anderson et al. (2006)
Early (1–3 h) cytokine response to anti-CD3 injection	Required	NKT?	Anderson et al. (2006)
Cardiac allograft rejection	Mildly required; cooperates with PKCα	Th1, CTL	Gruber et al. (2009)
	Required		Manicassamy et al. (2008)
Coxsackie B3-induced autoimmune myocarditis	Dispensable	Th1	Marsland et al. (2007)
α-myosin/CFA-induced experimental autoimmune myocarditis	Required	Th17	Marsland et al. (2007)
Autoimmune colitis, EAE	Required	Th17, Th1	Anderson et al. (2006), Salek-Ardakani et al. (2005), Tan et al. (2006)
Methylated BSA-induced arthritis	Required	Th1, Th2, B cells	Healy et al. (2006)
Concanavalin A-induced autoimmune hepatitis	Required	NKT	Fang et al. (2012)

⋆2×10^3 CFU LM-Ova.
†5×10^4 CFU LM-Ova.
‡Ova/CFA immunization s.c.
§Ova/alum immunization i.p.

responses. Consistent with these findings, mouse T cell responses triggered by immunization with a protein antigen plus an LPS adjuvant (a TLR4 agonist) were relatively well preserved in the absence of PKCθ (Valenzuela et al., 2009). The in vitro differentiation of *Prkcq*$^{-/-}$ Th1 cells is moderately impaired (Marsland et al., 2004; Salek-Ardakani et al., 2004), most likely due to the lack of such innate immunity signals in culture. However, the ability of innate immunity signals to bypass the requirement for protective responses to pathogens may not be generalizable or absolute. In fact, studies demonstrated that the immune response against two unicellular protozoan parasites, *Toxoplasma gondii* (Nishanth et al., 2010) or *Plasmodium berghei* (Ohayon et al., 2010) was impaired in *Prkcq*$^{-/-}$ mice, reflecting defects in both Th1 and Th2 cytokines. The primary defect in these infections may lie in impaired activation of innate TLR signaling pathways (Griffith et al., 2007; Yarovinsky et al., 2005), with the subsequent adaptive immunity defect representing a secondary phenomenon. Therefore, it remains to be determined whether unicellular parasites represent a special case where immunity and subsequent pathology require PKCθ and/or whether some ligands that stimulate the innate immune system are incapable of rescuing its deletion.

Using several models of Th2-mediated immune responses such as allergic lung inflammation and immunity to parasites, it has been clearly demonstrated that in vivo Th2 responses as well as Th2 differentiation in vitro are critically dependent on PKCθ (Marsland et al., 2004; Salek-Ardakani et al., 2004). This dependence almost certainly reflects the importance of PKCθ in upregulating the expression of GATA-3, the master transcription factor for Th2 development (Stevens et al., 2006). Although several studies demonstrated that PKCθ plays a less important role in Th1 responses (Marsland et al., 2004; Salek-Ardakani et al., 2004), several recent studies demonstrated that *Prkcq*$^{-/-}$ mice were resistant to the development of several experimental autoimmune diseases that are generally considered to represent reliable models of their human counterparts, including experimental autoimmune encephalomyelitis (EAE), adjuvant-induced arthritis, and colitis (Anderson et al., 2006; Healy et al., 2006; Salek-Ardakani et al., 2005; Tan et al., 2006). These autoimmune disease models, which were once considered to be induced by Th1 cells, are now known to be largely mediated by pathogenic Th17 cells. Indeed, *Prkcq*$^{-/-}$ CD4^{+} T cells display impaired in vitro differentiation into Th17 cells (Anderson et al., 2006; Kwon et al., 2012; Salek-Ardakani et al., 2005). The mechanistic basis for the regulation of Th17 differentiation by PKCθ appears to involve the upregulation of

Stat3 expression, which, in turn, is dependent on NF-κB and AP-1 (Kwon et al., 2012), the two transcription factors that are well known to represent PKCθ targets.

More recent studies expanded the list of in vivo immune responses that are differentially regulated by PKCθ. Analysis of cardiac allograft rejection revealed that *Prkcq*$^{-/-}$ mice showed a mildly prolonged allograft survival (Gruber et al., 2009) or a severe defect in their ability to reject such allografts (Manicassamy et al., 2008). The difference between the two studies can most likely be explained by the different strategies used to generate the corresponding *Prkcq*$^{-/-}$ mice (Pfeifhofer et al., 2003; Sun et al., 2000), as discussed earlier. Of interest, combined deletion of *Prkcq* and *Prkca* genes in double knockout mice resulted in a more severe delay in allograft rejection, suggesting cooperation between these two PKC isoforms, most likely operating at the level of NFAT activation (Gruber et al., 2009). The other study (Manicassamy et al., 2008) additionally demonstrated that the defect in allograft rejection mediated by *Prkcq*$^{-/-}$ T cells can be rescued by transgenic expression of the antiapoptotic protein Bcl_{xL} indicating that this defect is due, at least in part, to the poor survival of *Prkcq*$^{-/-}$ T cells. This conclusion is consistent with the well-established role of PKCθ as a T cell survival factor (Barouch-Bentov et al., 2005; Manicassamy, Gupta, et al., 2006; Saibil, Jones, et al., 2007) that regulates pro- and antiapoptotic Bcl2 family members in opposite ways, respectively. Of interest, a blocking (antagonistic) anti-CD28 antibody was found to enable long-term survival of heart allografts across a complete MHC mismatch, and this effect was associated with impaired early TCR signaling events, including PKCθ activation (Jang et al., 2008).

Allogeneic bone marrow transplantation (BMT) is commonly used as therapy for hematopoietic malignancies, and it relies on the T cell-dependent graft-*versus*-leukemia (GvL) response to eradicate residual tumor cells. However, GvHD elicited by alloreactive donor T cells that recognize mismatched recipient's histocompatibility antigens can cause severe damage to hematopoietic and epithelial tissues, and is often a potentially lethal complication of allogeneic BMT. Hence, strategies to eliminate the deleterious effects of GvHD and preserve the beneficial GvL response are highly desirable, but have been proven extremely difficult to achieve. A recent interesting publication reported that PKCθ was required for alloreactivity and GvHD induction, but was dispensable for the induction of a GvL response after BMT in mice (Valenzuela et al., 2009). In contrast, another study demonstrated an important role for PKCθ in the immune response

to de novo arising leukemias induced by Moloney murine leukemia virus in mice (Garaude et al., 2008). This role reflected the importance of PKCθ in both the activation of tumor-specific T cells as well as in the fitness of the growing leukemic cells. The latter effect is consistent with the critical role of PKCθ in T cell survival, including T cell leukemias (Villalba & Altman, 2002). Thus, unlike pathogen infections, where PKCθ is dispensable for immune pathogen clearance, elimination of danger signals represented by growing tumors appears to require PKCθ, potentially due to the absence of TLR ligands, which can compensate for the lack of PKCθ, on tumor cells.

Although the function of PKCθ was studied nearly exclusively in T cells, some studies also addressed its role in NK and natural killer T (NKT) cells, two T cell-related innate immune cells that are activated rapidly in response to danger signals such as those presented by tumor cells or virus-infected cells. PKCθ is expressed in NK cells (Balogh et al., 1999; Vyas et al., 2001) and, similar to T cells (see section 5 below), it translocates to the NK cell immunological synapse (IS) upon activation by MHC class I-deficient target cells (Davis et al., 1999). PKCθ was found to be important for NK cell-mediated surveillance of tumor cells and virus-infected cells via several potential mechanisms that may reflect its requirement for NK production of cytokines such as IFNγ and TNFα (Page et al., 2008; Tassi et al., 2008), NK cell degranulation (Aguilo et al., 2009), or induction of FasL (Pardo et al., 2003; Villalba et al., 1999; Villunger et al., 1999). A potential mechanism for a role of PKCθ in NK (and CTL) degranulation could involve the phosphorylation of Wiskott–Aldrich Syndrome protein (WASp)-interacting protein (WIP) during NK cell activation (Krzewski et al., 2006) since WIP and WASp are components of the cellular machinery that regulates the actin cytoskeleton, a process important for cell polarization and directional cytokine and lytic granules secretion (Krzewski et al., 2006). Other hypothetical mechanisms that may underlie the importance of PKCθ in tumor surveillance are plausible as well. The role of PKCθ in NK cell function has recently been reviewed in detail (Anel et al., 2012).

Prkcq$^{-/-}$ mice also display defects in the development and function of NKT cells (Fang et al., 2012; Schmidt-Supprian et al., 2004). The requirement of PKCθ for the thymic development of NKT cells most likely reflects the critical role of NF-κB, a known PKCθ target, in NKT development (Schmidt-Supprian et al., 2004). Fang et al. used an in vivo model of concanavalin A (ConA)-induced acute hepatitis, an inflammatory response known to be mediated by rapidly activated NKT cells, to study the role

of PKCθ. They found that *Prkcq*$^{-/-}$ mice were resistant to acute hepatitis, reflecting a requirement of PKCθ in both NKT cell development (resulting in reduced NKT cell numbers in the periphery) and activation (Fang et al., 2012). Thus, ConA- or NKT-specific lipid ligand-stimulated *Prkcq*$^{-/-}$ NKT cells displayed impaired IFNγ, TNFα and IL-6 production, and this defect was most likely intrinsic to the NKT cells.

5. PKCθ AND THE IMMUNOLOGICAL SYNAPSE

When the TCR on CD4$^+$ or CD8$^+$ T cells is engaged by antigen-presenting cells (APCs) that present a complex of peptide and MHC class II molecule, or by target cells displaying a peptide antigen bound to MHC class I molecule, respectively, the T cells go through a complex and dynamic process, whereby its surface proteins, plasma membrane (PM) lipids, the actin cytoskeleton, and intracellular signal mediators undergo spatial and temporal reorganization to form an IS in the contact area between the T cells and the APCs (or target cells). This IS acts as platform for signal initiation and termination (Bromley et al., 2001; Dustin, 1997; Dustin et al., 2001; Dustin & Cooper, 2000; Grakoui et al., 1999; Lee et al., 2003; Vardhana et al., 2010), where proteins and lipids segregate into distinct IS subdomains known as the central supramolecular activation cluster (cSMAC), peripheral SMAC (pSMAC), and distal SMAC (dSMAC) (Monks et al., 1998). The use of high-resolution imaging techniques such as total internal reflection fluorescence microscopy, which allows imaging of protein localization in live cells and in real time, revealed that upon T cell engagement by APCs and recognition of antigen, microclusters containing TCRs and additional signaling molecules continuously form at the periphery of the IS, whereupon they migrate centripetally into the center of the IS (Campi et al., 2005; Saito & Yokosuka, 2006; Varma et al., 2006; Yokosuka et al., 2005). Formation of these microclusters precedes the organization of a mature IS, and they represent a site for antigen recognition and T cell activation (Saito & Yokosuka, 2006). One of the most prominent discoveries about PKCθ was the finding that upon antigen stimulation, PKCθ translocates at a high stoichiometry into the cSMAC in the IS and, furthermore, that the level of its translocation positively correlated with the strength of the TCR signal (Monks et al., 1998; Monks et al., 1997). At the time of this discovery, it was thought that PKCθ was the only T cell-expressed PKC family member to translocate to the IS, but very recent studies revealed that two other nPKCs, PKCε and η, and perhaps even a few more (Kong

and Altman, 2013) also translocate to the IS, albeit not specifically to the cSMAC but, rather, in a diffuse pattern all over the IS (Quann et al., 2011; Singleton et al., 2011).

As a result of DAG formation in the PM following receptor-induced, PLCγ-mediated hydrolysis of membrane inositol phospholipids, most cPKCs and nPKCs are recruited to the PM via their tandem C1 domains (Newton, 1997; Nishizuka, 1995). However, PKCθ is unique in its highly selective localization to the cSMAC, suggesting that in addition to the high local concentration of DAG at the T cell IS (Spitaler et al., 2006), which mediates PKCθ (and perhaps other PKCs) recruitment to the IS in general, but not to the cSMAC specifically (Carrasco & Merida, 2004; Spitaler et al., 2006), another mechanism exists to direct PKCθ specifically to the cSMAC. This hypothetical mechanism remained an enigma for a long time, as did the apparent paradox that PKCθ, which is thought to sustain TCR signaling in the IS for hours, is localized in an IS subdomain (the cSMAC), where TCR signaling complexes are degraded and signaling is terminated (Lee et al., 2003; Vardhana et al., 2010). Findings that CD28, but not other costimulatory receptors, is essential for PKCθ localization at the cSMAC (Huang et al., 2002; Sedwick et al., 1999), and that PKCθ colocalizes with CD28 in TCR-dependent microclusters and, later, in a cSMAC subregion distinct from the TCR-high subregion (Tseng et al., 2005; 2008; Yokosuka et al., 2008) provided a potential resolution to these unresolved question. Yokosuka et al. were the first to demonstrate a physical association (revealed by coimmunoprecipitation) between PKCθ and CD28 (Yokosuka et al., 2008), and this finding was extended and further explored by us under conditions of specific antigen stimulation (Kong et al., 2011). We demonstrated that the V3 (hinge) domain of PKCθ is required and sufficient for the recruitment of PKCθ to the cSMAC, reflecting an indirect physical association between the V3 domain and the cytoplasmic tail of CD28. The intermediate protein in this trimolecular complex is the Lck tyrosine kinase, which associates via its SH2 and SH3 domains with a distal tyrosine-phosphorylated motif in the CD28 tail and with an evolutionary conserved proline-rich motif in the PKCθ V3 domain (which is not found in other PKCs), respectively (Kong et al., 2011). The PKCθ–CD28 association was essential for downstream PKCθ-dependent functions as V3 mutations that abolished this interaction, or ectopic expression of the isolated PKCθ V3 domain, which functioned in a dominant negative manner to disrupt the endogenous PKCθ–CD28 association, disrupted the activation of NF-κB and

the differentiation of naïve T cells into Th2 or Th17, but not Th1 cells (Kong et al., 2011). This effect on Th differentiation is fully consistent with the studies described earlier, which documented the requirement, or lack thereof, of PKCθ for the differentiation of these Th subsets.

Other mechanisms may also contribute to the IS and cSMAC localization of PKCθ. First, TCR/CD28-induced autophosphorylation of Thr-219 in the regulatory domain of PKCθ was important for IS localization as well as for NF-κB activation (Thuille et al., 2005). Our recent study (Kong et al., 2011) is consistent with this correlation between the IS localization and function of PKCθ. Second, intact catalytic activity was also important since deletion of the catalytic domain or mutation of several PKCθ phosphorylation sites, including Thr-538, which is essential for the catalytic competence of the kinase, abolished or greatly reduced its IS recruitment following antigen stimulation (Cartwright et al., 2011). Third, a very recent study demonstrated that PKCε and PKCη are also recruited to the IS in a diffuse manner. Interestingly, the recruitment of these two nPKCs preceded that of PKCθ to the cSMAC and, in fact, seemed to be obligatory since RNA-mediated combined knockdown of these two PKCs reduced the subsequent IS/cSMAC localization and function of PKCθ (Quann et al., 2011). Furthermore, PKCθ was found to be important for the organization of the microtubule organizing complex under the IS in this study. Thus, a PKC cascade may operate in T cells to promote the unique localization and function of PKCθ, at least in T effector (Teff) cells. Additional signaling proteins that appear to participate in the regulation of PKCθ cellular localization and function include the ERK-activating MEK kinase (Praveen et al., 2009), phosphatidylinositol 3-kinase (PI3K) (Praveen et al., 2009; Villalba et al., 2002) and Vav (Dienz et al., 2000; 2003; Villalba et al., 2000; 2002).

The stable IS is a symmetrical ("bull's eye") structure that forms upon T cells contact with APCs. However, T cells can undergo transient interactions with APCs, in which disengagement from the APC and the subsequent T cell motility result in breaking of the IS symmetry and formation of an unstable, nonsymmetrical synapse termed *kinapse* (Dustin, 2008). The kinapse would then reassemble into a stable, symmetrical synapse when the T cell serially engages a new APC. Naive T cells encountering APCs were found to undergo cycles of stable IS formation and autonomous T cell migration associated with kinapse formation, which was driven by PKCθ (Sims et al., 2007). In these motile T cells, PKCθ was localized to the F-actin-dependent peripheral pSMAC.

Consistent with an important role of PKCθ in promoting destabilization of the IS and formation of a kinase, *Prkcq*$^{-/-}$ T cells formed hyperstable IS in vitro and in vivo; conversely, the WASp promoted the formation of a stable IS (Sims et al., 2007). Thus, opposing effects of PKCθ and WASp control IS stability through pSMAC symmetry breaking and reformation.

Along the same line, PKCθ was reported to destabilize the IS in CD4$^+$ CTLs since a selective PKCθ inhibitor increased IS stability and sensitivity of specific target cell lysis (Beal et al., 2008). Conversely, disruption of the pSMAC by treatment with anti-LFA-1 antibody destabilized the CD8$^+$ CTL IS and decreased target cell sensitivity to lysis. This study also demonstrated that CD4$^+$ CTLs form a less stable IS with target cells than their CD8$^+$ counterparts, which correlate with relatively reduced lytic efficiency. These results suggest that formation of a stable pSMAC, which is inhibited by PKCθ, functions to confine the released lytic molecules at the synaptic interface and to enhance the effectiveness of target cell lysis. However, evidence also exists indicating that PKCθ promotes IS stability. Thus, PKCθ was reported to activate the β2 integrin LFA-1 (i.e. increase its avidity for its ligand ICAM-1) downstream of the TCR by phosphorylating the guanine nucleotide exchange factor Rap-GEF2, an activator of the small GTPase Rap1 (Letschka et al., 2008). Additional studies will be required to settle this apparent discrepancy.

As discussed earlier, T cell activation leads to a segregation of PM domains to form TCR signaling clusters and eventually the IS. At these T cell activation sites, protein networks reside in PM regions that contain highly ordered lipids such as cholesterol and sphingomyelin in subdomains broadly referred to as lipid rafts. These lipid rafts are implicated in signaling from the TCR and in localization and function of proteins residing proximal to the TCR, and they localize at the IS (Harder et al., 2007; Kabouridis & Jury, 2008). Despite many studies on the role of lipid rafts in T cell activation, their importance in this process is still somewhat controversial (Kenworthy, 2008), and TCR microcluster formation is, in fact, independent of lipid raft clustering (Saito & Yokosuka, 2006). Imaging analysis demonstrated that lipid rafts preferentially accumulate in the cSMAC. However, quantitative analyses indicated that the level of lipid rafts recruitment to the cSMAC is relatively small, suggesting that rearrangement of lipid rafts from the pSMAC into the cSMAC can account for this accumulation (Burack et al., 2002).

We found that T cell stimulation by antireceptor antibodies or by peptide–MHC complexes induces translocation of PKCθ to membrane lipid rafts, which localized to the IS. This translocation was mediated by the regulatory domain of PKCθ, was dependent on Lck (but not ZAP-70) kinase, and a PKCθ–Lck complex was present in the lipid rafts (Bi et al., 2001). The isolated catalytic domain of PKCθ did not partition into rafts and was incapable of activating NF-κB, but addition of an Lck-derived acylation signal, which targeted the catalytic domain into lipid rafts, restored these functions. Thus, physiological T cell activation translocates PKCθ to rafts, and this translocation is important for its function (Bi et al., 2001).

6. PKCθ, CD28 COSTIMULATION, AND T CELL ANERGY

T cell anergy is an important mechanism of peripheral immune tolerance, whereby T cells primed by antigen fail to respond to restimulation with the same antigen (Fathman & Lineberry, 2007; Schwartz, 2003). TCR signaling events are aberrant in anergic T cells, and the underlying mechanisms are complex (Saibil et al., 2007). However, one dominant theme that has emerged from recent studies is the importance of the Ca^{2+} signaling pathway involving NFAT activation in anergy induction (Heissmeyer et al., 2004; Heissmeyer & Rao, 2004; Macian et al., 2002; Macian et al., 2004). Thus, T cell anergy ensues when NFAT is activated by partial TCR signals in the absence of AP-1 and/or NF-κB activation, reflecting the induction of a unique anergy-associated gene program (Heissmeyer et al., 2004; Macian et al., 2002), which involves the upregulation of E3 ubiquitin ligases and the resulting degradation of early signal transducing proteins (Fathman & Lineberry, 2007; Heissmeyer & Rao, 2004; Macian et al., 2004).

Based on the "two-signal hypothesis", which states that productive T cell activation requires a TCR signal (signal 1) and an additional costimulatory signal (signal 2) (Bretscher & Cohn, 1968), it was found that provision of a TCR signal in the absence of costimulation induces T cell anergy (Harding et al., 1992; Jenkins et al., 1990). Subsequently, CD28 was identified as the major costimulatory receptor in naïve T cells (Harding et al., 1992). In this context, it is interesting to note that many of the TCR signaling events that are impaired in anergic T cells, such as the activation of Ras, MAP kinases, AP-1 and NF-κB (Fathman & Lineberry, 2007; Saibil, Deenick, et al., 2007; Schwartz, 2003), represent downstream targets

of PKCθ, raising the intriguing possibility that PKCθ plays a key role in determining the balance between productive T cell activation and anergy. Several lines of evidence support this notion. First, it is now clear that PKCθ integrates signals from both the TCR and CD28, a requirement for its functional activity (Coudronniere et al., 2000) and proper localization in the T cell IS (Huang et al., 2002; Kong et al., 2011; Sedwick et al., 1999). Second, *Prkcq*$^{-/-}$ T cells display an anergic phenotype upon antigen challenge, similar to that of *Cd28*$^{-/-}$ mice (Berg-Brown et al., 2004). And, third, a blocking anti-CD28-specific antibody was reported to induce long-term heart allograft survival by suppressing the PKCθ–JNK signaling pathway (Jang et al., 2008). Given the less severe inhibitory effect of *Prkcq* deletion on the Ca^{2+}-NFAT signaling pathway relative to the AP-1 and NF-κB pathways, it is therefore conceivable that in the absence of PKCθ, there would be sufficient residual NFAT activation but nearly absent AP-1 and NF-κB activation, conditions that would favor the induction of T cell anergy (Heissmeyer et al., 2004). Hence, selective inhibition of PKCθ function could potentially achieve the beneficial effect of inducing anergy (tolerance) to organ and bone marrow transplants.

7. UNIQUE FUNCTION OF PKCθ IN REGULATORY T CELL DEVELOPMENT AND FUNCTION: THE YANG

Tregs play an indispensable role in maintaining immune homeostasis and immunological unresponsiveness to self-antigens, as well as in suppressing excessive immune responses deleterious to the host, such as autoimmune and autoinflammatory disorders, allergy, acute and chronic infections, cancer, and metabolic inflammation. Tregs are generated in the thymus as a functionally mature subpopulation of T cells termed natural Tregs (nTregs) and can also be induced from naive T cells in the periphery by an appropriate cytokine milieu to differentiate into induced Tregs (iTregs). Foxp3 serves as an essential master transcription factor that determines Treg lineage specification (Josefowicz et al., 2012; Josefowicz & Rudensky, 2009; Rudensky, 2011; Sakaguchi et al., 2008).

PKCθ was found to be required for the thymic development of nTregs (Gupta et al., 2008; Schmidt-Supprian et al., 2004). This requirement is not absolute, however, since *Prkcq*$^{-/-}$ mice still have ~20% of the nTregs found in wild-type mice. Moreover, ex vivo Tregs isolated from *Prkcq*$^{-/-}$ mice display intact suppressive activity (Gupta et al., 2008) (K.-F. Kong, unpublished data). The requirement of PKCθ reflected its important role in

activating the NF-κB signaling pathway because, similar to PKCθ deletion, deletion of IKKβ and Bcl10, two critical components in the canonical NF-κB pathway, reduced nTreg development (Schmidt-Supprian et al., 2004). The importance of NF-κB in Treg development is also evident from the finding that the transcription factor c-Rel initiates *Foxp3* transcription in thymic Treg precursors (Hori, 2010). The nTreg development defect in *Prkcq*$^{-/-}$ mice was not related to a missing survival signal since transgenic expression of the antiapoptotic survival protein Bcl_{xL} could not restore the Treg cell population in these mice (Gupta et al., 2008). In addition, CN-Aβ-deficient mice also had a decreased Treg cell population similar to that observed in *Prkcq*$^{-/-}$ mice, suggesting that NFAT also plays an important role in nTreg development (Gupta et al., 2008).

One prominent Treg-mediated suppressive mechanism is dependent upon its contact with APCs. This physical contact promotes the formation of a specialized signaling platform, the IS, at the Treg–APC interface (Sakaguchi et al., 2008; Sarris et al., 2008; Zanin-Zhorov et al., 2010). A recent study explored the characteristics of the Treg IS and, surprisingly, reported the intriguing finding that in contrast to Teff cells, PKCθ is excluded from the Treg IS and, instead, it localizes to the distal pole in human Tregs (Zanin-Zhorov et al., 2010). Furthermore, a selective small molecule PKCθ inhibitor (C20) enhanced the suppressive activity of Tregs, implying a negative regulatory role for PKCθ on Treg function. Similarly, pharmacological inhibition of NF-κB also increased human Treg suppressive activity, suggesting that PKCθ targets NF-κB in a pathway leading to inhibition of Treg function. This contrasts with the positive regulatory function of the NF-κB pathway in thymic Treg development (Hori, 2010; Schmidt-Supprian et al., 2004). Pharmacological inhibition of PKCθ protected Tregs from inactivation by TNFα, rescued the defective activity of Tregs from rheumatoid arthritis (RA) patients, and enhanced protection of mice from inflammatory colitis (Zanin-Zhorov et al., 2010). However, the PKCθ inhibitor abolished the ability of human Tregs to proliferate in vitro in response to anti-CD3 plus CD28 antibodies in the presence of high IL-2 concentrations (Zanin-Zhorov et al., 2010; Zanin-Zhorov et al., 2011). Our preliminary finding that a dominant negative PKCθ V3 domain, which inhibits the differentiation of Th2 and Th17 cells (Kong et al., 2011), enhances the in vitro differentiation of naïve T cells into FoxP3^{+} Tregs (K. F. Kong & E. Y. Zhang, unpublished data) supports the inhibitory role of PKCθ in Treg function. Another, indirect support for this notion comes from a very recent study, which demonstrated that

embryonic stem cells-derived factors, which have been known to modulate immune activation, inhibited the phosphorylation of PKCθ and the activation of its target, NF-κB, in Teff cells, while at the same time, upregulating Treg markers such as FoxP3, TGFβ and IL-10 in $CD4^+CD25^+$ cells (Mohib et al., 2012).

Another very recent study also addressed the role of PKCθ in Treg differentiation (Ma et al., 2012). These authors reported that PKCθ-mediated signals inhibit iTreg differentiation in vitro via an Akt-Foxo1/3A pathway. This conclusion was based on findings that TGFβ-induced iTreg differentiation was enhanced in *Prkcq*$^{-/-}$ T cells or in wild-type T cells treated with a selective PKCθ inhibitor, and that *Prkcq*$^{-/-}$ T cells displayed reduced Akt kinase activity. Furthermore, knockdown or overexpression of the Akt targets Foxo1 and Foxo3a inhibited or promoted the iTreg differentiation of *Prkcq*$^{-/-}$ T cells, respectively. By contrast, we found that naïve T cells from *Prkcq*$^{-/-}$ mice displayed a severely impaired differentiation into $Foxp3^+$ Treg cells when cultured under similar conditions to those used by Ma et al. (2012), i.e. anti-CD3/CD28 antibody stimulation in the presence of TGFβ and IL-2 (K.-F. Kong, unpublished data) suggesting that PKCθ is, in fact, indispensable for the in vitro differentiation of $CD4^+Foxp3^+$ T cells. The reason for these apparently contradictory findings is unclear but it is important to note that important caveats need to be taken into consideration when assessing the effect of pharmacological PKCθ inhibitors or *Prkcq* gene deletion on the differentiation of Tregs (or T cells in general): in the first case, small molecule kinase inhibitors do not have absolute selectivity and, therefore, functional effects of PKCθ inhibition could conceivably be due to the inhibition of other kinases. In the second case, embryonic deletion could affect T cell developmental processes, and these effects could be carried over to the peripheral T cells. Hence, it would be useful to generate conditional *Prkcq* gene knockout mice where PKCθ expression is abolished post T cell development, i.e. only in the mature peripheral T cells.

Another issue that merits consideration when studying the function of PKCθ in Treg development, differentiation, and function has to do with the role of CD28 in Tregs, given the importance of this costimulatory receptor in the IS localization and function of PKCθ in Teff cells. In contrast to thymic Treg development that requires high-affinity and -avidity TCR interaction together with a CD28 costimulatory signal, peripheral Treg induction depends on suboptimal TCR stimulation together with TGFβ, but in the absence of CD28 costimulation (Curotto de Lafaille & Lafaille,

2009; Josefowicz & Rudensky, 2009). In fact, CD28 signals can inhibit iTreg differentiation (Ma et al., 2012; Zanin-Zhorov et al., 2010), most likely reflecting the importance of CD28 in promoting activation of NF-κB (Coudronniere et al., 2000), consistent with the negative effect of NF-κB on iTreg differentiation (Zanin-Zhorov et al., 2010).

8. PKCθ IN HUMAN DISEASE

The establishment of *Prkcq*$^{-/-}$ mice (Pfeifhofer et al., 2003; Sun et al., 2000) made it possible to systematically dissect the critical functions of PKCθ at the molecular, cellular and in vivo levels under physiological and pathological conditions. However, the potential role of PKCθ in the pathogenesis of human diseases represents a much more challenging question. Nonetheless, PKCθ has consistently been reported to be associated with several human diseases, including autoimmune diseases and cancer.

Recent genome-wide association studies, which compare single nucleotide polymorphisms (SNPs) between thousands of diseased and healthy individuals followed by powerful statistical analyses, have identified specific SNPs within the *Prkcq* locus that are significantly associated with type 1 diabetes (T1D), RA, and celiac disease (Cooper et al., 2008; Raychaudhuri et al., 2008; Stahl et al., 2010; Zhernakova et al., 2011). For example, the SNP rs947474 has been reproducibly reported as a risk factor for the development of T1D (Cooper et al., 2008; Reddy et al., 2011). On the other hand, the C or G single-nucleotide variation at rs4750316 is consistently associated with RA (Raychaudhuri et al., 2008; Stahl et al., 2010). These novel findings provide a framework for formulating tangible hypotheses and testable models in order to understand the PKCθ-dependent molecular pathways pertinent to human diseases. For example, the T1D susceptibility associated with SNP rs947474, which is located 78 kb downstream of the *Prkcq* gene, is positioned within the gene regulatory region. Incidentally, a comprehensive genome-wide mapping study using ChIP followed by deep sequencing (ChIP-seq) revealed that the same SNP lies within a vitamin D receptor (VDR)-binding site (Ramagopalan et al., 2010). The VDR is a transcription factor, which, upon binding to its ligand, exerts pleiotropic biological effects on immune cells, including T cells (Baeke et al., 2010). Thus, this disease-associating SNP could possibly affect the occupancy and/or function of the VDR. With this knowledge, it would be feasible and interesting to examine whether the single nucleotide variation at rs947474 can modulate the expression

and/or function of human PKCθ and, as a result, increase the risk of developing T1D.

Another intriguing development concerning the association of PKCθ with human disease has recently emerged in cancer studies. Gastrointestinal stromal tumors (GISTs) represent a specific group of tumors involving the mesenchymal tissues of the gastrointestinal tract. Gain-of-function mutations in the *c-Kit* protooncogene that result in its constitutive activity account for about 85% of cases and, hence, c-Kit expression is the standard marker for GIST diagnosis (Hirota et al., 1998). Treatment of GIST patients with the tyrosine kinase inhibitor, Imatinib, is effective, although its long-term use can lead to drug resistance (Gschwind et al., 2004). In a small subset of GIST patients, the expression of c-Kit is less prominent and, therefore, in an effort to identify new markers for c-Kit-negative GIST, several groups found that PKCθ was expressed in all forms of GISTs, but not in other mesenchymal or epithelial tumors, including non-GIST c-Kit-positive tumors. Thus, PKCθ can serve as a sensitive and specific marker for GISTs (Blay et al., 2004; Debiec-Rychter et al., 2004; Duensing et al., 2004). Subsequent studies in different cancer centers have corroborated this finding (Lee et al., 2008; Motegi et al., 2005). Does the aberrant expression of PKCθ in GISTs play a role in the development of these tumors? In fact, in vitro experiments demonstrated that PKCθ acts upstream to regulate the expression of c-Kit since knockdown of PKCθ in GIST cell lines using RNA interference caused a reduction of c-Kit expression, inhibition of the PI3K/Akt signaling pathway, upregulation of the cyclin-dependent kinase inhibitors p21 and p27, cell arrest at the G1 phase of the cell cycle, and apoptosis (Ou et al., 2008). Hence, these findings suggest that PKCθ could promote GIST development and, therefore, its inhibition could potentially be therapeutically beneficial.

The link between the aberrant expression of PKCθ and GIST might represent merely the tip of the iceberg. Earlier preliminary studies and a recent more focused report indicate that the PKCθ could also be detected in Ewing's sarcoma, which is a rare group of bone neoplasms affecting mainly children and adolescents (Blay et al., 2004; Kang et al., 2009). In a small subset of Ewing's sarcomas, PKCθ appeared to have a characteristic dot-like localization, suggesting its utility as a prognostic marker (Kang et al., 2009). However, this interesting observation warrants further careful study. PKCθ was also implicated as a critical regulator of c-Rel-driven mammary tumorigenesis, as PKCθ activation

inhibited the FOXO3a/ERα/p27Kip1 axis that normally maintains an epithelial cell phenotype and induces c-Rel target genes, thereby promoting proliferation, survival, and more invasive breast cancer (Belguise & Sonenshein, 2007).

Other examples for the potential involvement of PKCθ in human disease include its reported role in mediating insulin resistance (Griffin et al., 1999; Itani et al., 2000; Kim et al., 2004; Serra et al., 2003), the high-level expression of GLK, a direct PKCθ-activating kinase, in T cells of systemic lupus erythematosus patients (Chuang et al., 2011) and, as mentioned earlier, the restoration of the impaired Treg activity in RA patients by a PKCθ inhibitor (Zanin-Zhorov et al., 2010). Altogether, these emerging reports on the association between PKCθ and human diseases highlight the need to better understand the function of this enzyme in the human immune system and to seek approaches that could inhibit its function in humans (see section 9 below).

9. IS PKCθ A PROMISING DRUG TARGET?

Since its discovery, PKCθ has garnered considerable amount of attention as a potential therapeutic target (Baier, 2003; Baier & Wagner, 2009). Previous sections of this review have provided several strong arguments that support, at least from a theoretical standpoint, a strong case for considering PKCθ as an attractive drug target for selective T cell immunosuppression. Perhaps the strongest argument is provided by the now well-established documentation of the selective requirement of PKCθ in T cell-dependent immune responses (Table 6.1). Particularly intriguing and exciting are the findings that PKCθ is critical for harmful immune responses, namely, Th2-meditaed allergies, Th17-mediated autoimmune diseases, and GvHD, but is dispensable for beneficial immune responses such as protection against pathogen infection mediated by Th1 cells and CTLs and GvL responses in BMT recipients. Of particular interest are the findings that pathogenic T cells depend on PKCθ, whereas Tregs are, in fact, negatively regulated by it. Thus, strategies to inhibit PKCθ would be expected to achieve a synergistic outcome of simultaneously inhibiting inflammatory T cells and promoting Treg function, a highly desirable scenario in autoimmune diseases and transplantation. However, promotion of Treg function and inhibition of effector T cell function represent a double-edged sword because they would be desirable in, e.g. autoimmune diseases, but not in tumor-specific T cell responses.

Second, based on studies reviewed earlier, it is likely that inhibition of PKCθ would also promote anergy induction by preventing (or diminishing) the activation of AP-1 and NF-κB, two transcription factors that control the balance between anergy (induced by NFAT activation alone) and productive T cell activation in favor of the latter. This scenario can be contrasted with CN inhibitors, which are widely used for immunosuppression, because, unlike PKCθ, which almost certainly antagonizes anergy induction, NFAT (the target of CN inhibitors) is required for maintaining anergy to organ transplants. Hence, PKCθ inhibition could be expected to interfere with transplant rejection by donor Teff cells and, at the same time, enable anergy against the transplant to become stably established. Third, PKCθ provides a survival signal, particularly to activated and potentially pathogenic T cells as well as to leukemic T cells. Therefore, it is conceivable that PKCθ inhibition would promote the apoptosis of pathogenic T cells and perhaps even T cell leukemias. Last but not least, PKCθ has a relatively narrow range of tissue distribution with predominant expression in T cells and, therefore, minimal toxic side effects of PKCθ inhibitory drugs can be expected in tissues other than T cells. This expectation is supported by the generally intact health status and fertility of *Prkcq*$^{-/-}$ mice, and is, again, in sharp contrast to the considerable toxicity of CN inhibitors, which reflects the ubiquitous tissue distribution and functions of NFAT.

On the other hand, it is also important to consider whether highly selective inhibition of PKCθ alone would be sufficient to achieve desirable and effective therapeutic effects. This question arises because of the possibility of functional redundancy among T cell-expressed PKCs. Despite the fact that *Prkcq*$^{-/-}$ T cells display severe activation defects, which are somewhat milder in the mice generated by Baier et al. (Pfeifhofer et al., 2003), and the nearly absolute requirement of PKCθ in certain murine immune responses (e.g. Th2, Th17), there is substantial evidence for cooperation and functional redundancy between PKCθ and other T cell-expressed PKCs (Baier & Wagner, 2009). The following are several examples for this redundancy: First, PKCε enhances NF-κB, NFAT and AP1 signaling pathways leading to IL-2 expression (Genot et al., 1995; Szamel et al., 1998), promotes proliferation of human CD4$^+$ T cell by attenuating the inhibitory effects of TGFβ1 (Mirandola et al., 2011), and also has an antiapoptotic effect in T cells (Bertolotto et al., 2000; Villalba et al., 2001). Second, PKCα cooperates with PKCθ to downregulate TCR expression (von Essen et al., 2006) and to promote certain aspects of T cell activation,

e.g. alloimmune responses and IFNγ production (Gruber et al., 2009); PKCα is also required for Th1-dependent IgG2a/2b antibody responses (Pfeifhofer et al., 2006). Third, double-knockout mice deficient in both PKCη and PKCθ have a significant defect in T cell development, which is not observed in the corresponding single-knockout mice and, furthermore, PKCη promotes the activation of mature $CD8^+$ T cells and homeostatic proliferation (Fu et al., 2011). Fourth, PKCβ positively regulates T cell migration (Volkov et al., 1998; Volkov et al., 2001), expression of the activation marker CD69 and secretion of IL-8 (Cervino et al., 2010), and IL-2 exocytosis in T cells (Long et al., 2001). Finally, PKCζ has been shown to control Th2 cell function and allergic airway inflammation (Martin et al., 2005). Thus, the potential contribution of other PKC family members to the activation, differentiation and function of T cells (and other immune cells) has to be taken into account when considering the development of PKCθ-based therapeutics for clinical use, especially given the fact that little is known about the functions of this enzyme in human T cells.

In view of the above considerations, it is not surprising that pharmaceutical drug companies have dedicated substantial efforts to identify and characterize small molecule inhibitors of PKCθ catalytic activity. Recent studies reported the development and characterization of compounds that display various degree of selectivity toward PKCθ (Cole et al., 2008; Cywin et al., 2007; Mosyak et al., 2007). These small molecules function as ATP competitive inhibitors, i.e. they bind to the ATP-binding pocket of the kinase. A majority of kinase inhibitors developed to date target this same site. However, because this site is conserved among kinases, it is difficult to obtain highly selective inhibitors. For example, imatinib, a Bcr-Abl kinase inhibitor that is used to treat chronic myelogenous leukemia patients, was found to inhibit several unrelated tyrosine kinases, and the concept of "multikinase" inhibition as a beneficial rather than an undesired effect is gaining some prominence (Kontzias, Laurence, Gadina, & O'Shea, 2012). Furthermore, since catalytic kinase inhibitors in current clinical use are ATP competitors, they need to be used at relatively high and potentially toxic concentrations in order to effectively compete with ATP, whose intracellular concentration is ~1 mM.

Among recent PKCθ inhibitors, the compound AEB071 (sotrastaurin) seems to have reached the most advanced development stage, and it has entered clinical trials in psoriasis and organ transplantation (Budde et al., 2010; Friman et al., 2011; Skvara et al., 2008). AEB071 inhibits not only

PKCθ, but also other novel (Ca^{2+}-independent δ, ε, and η; nPKC) and conventional (Ca^{2+}-dependent α and β; cPKC) members at subnanomolar to low nanomolar concentrations, with a 1000–10,000-fold lower selectivity for other kinases (Evenou et al., 2009; Skvara et al., 2008). It also effectively inhibited anti-TCR/CD28-stimulated human and mouse T cell proliferation and cytokine production (Evenou et al., 2009; Matz et al., 2010), as well as local GvHD and allograft rejection in rats and non-human primates (Bigaud et al., 2012; Kamo et al., 2011; Weckbecker et al., 2010), and was well tolerated. Consistent with the findings that PKCθ is dispensable for antiviral immunity (Berg-Brown et al., 2004; Giannoni et al., 2005; Marsland et al., 2005; Valenzuela et al., 2009), PKC inhibition with AEB071 did not lead to increased infections in renal transplant patients enrolled in a phase II clinical trial (Friman et al., 2011). However, it remains to be seen whether AEB071 will be sufficiently effective as a monotherapy. Since it reportedly does not inhibit Ca^{2+} signaling (Evenou et al., 2009), combination therapy with other immunosuppressive agents such as cyclosporine A at low, suboptimal concentrations (Bigaud et al., 2012; Budde et al., 2010; Evenou et al., 2009; Weckbecker et al., 2010) may be useful, provided it does not cause global, potentially harmful immunosuppression.

It has been argued that the ability of AEB071 to broadly inhibit PKCs underlies its inhibition of T cell activation since this broad activity prevents potential compensation by other PKC isoforms (Baier & Wagner, 2009; Friman et al., 2011). Indeed, as mentioned earlier, functional cooperation and partial redundancy between PKCθ and other PKCs, including PKCα (Gruber et al., 2009) has been demonstrated (Baier & Wagner, 2009; Pfeifhofer-Obermair et al., 2012). However, the finding that the combined deletion of PKCθ and PKCα primarily affects NFAT activation (Gruber et al., 2009) is inconsistent with findings that AEB071 does not inhibit NFAT activation (Evenou et al., 2009). Thus, some other PKC besides PKCθ or PKCα, or even a non-PKC kinase that is not inhibited by AEB071, may be important. Moreover, it is hard to predict whether potent inhibition of other PKC family members besides PKCθ may be beneficial by overcoming kinase redundancy or, conversely, may have the undesired effect of inducing global immunosuppression or some toxicity. In fact, adverse effects, particularly those affecting the gastrointestinal tract, were reported with higher incidence in renal transplant recipients in a phase II AEB071 clinical trial (Friman et al., 2011). Hence, it remains unclear whether lower selectivity toward PKC family members would represent a

therapeutic advantage or disadvantage, and further clinical trials are required to determine if the therapeutic benefits of AEB071 outweigh its side effects.

Given the potential toxicity and side effects of small molecule kinase inhibitors, there has recently been an increased interest in allosteric kinase inhibitors, i.e. compounds that do not bind to the catalytic pocket of the kinase but, rather, to another, regulatory region and, by doing so prevents kinase activation, most likely by interfering with a conformational change required for opening of the catalytic pocket and, hence, full activity (Lamba & Ghosh, 2012). Because allosteric inhibitors bind to much less conserved sites in kinases, they are likely to be much more selective and less toxic. Consideration of PKCθ as a potential target for allosteric inhibition requires that at least two criteria are met. First, the enzyme should contain a defined allosteric site that is necessary for its activation and downstream functions in order to ensure efficacy. Second, this allosteric site should be unique, i.e. have low sequence homology to other PKCs (or kinases in general) to ensure a high degree of specificity (with the proviso that exquisite specificity is indeed an advantage, as opposed to the potential benefits of "multikinase" inhibition). We propose that the proline-rich motif in the V3 domain of PKCθ, which we found recently to be essential for targeting it to the IS and the cSMAC, and enabling it to activate its downstream targets (Kong et al., 2011), meets these criteria. The V3 domain is the most divergent region among members of the PKC family, and the critical proline-rich motif is found only in PKCθ. V3 domains of PKCs were initially considered to represent a flexible hinge region for the "opening" of PKC and its change from a resting state to the active conformation for substrate binding and kinase activity (Steinberg, 2008). However, it is becoming clear that the hinge regions of PKCs have additional functions, including protein–protein interactions. Indeed, in addition to PKCθ, it has been reported that the G(D/E)E motif located in the V3 region of PKCα and PKCε is essential for the selective targeting of these isoforms (Quittau-Prevostel et al., 2004).

10. CONCLUSION AND FUTURE PERSPECTIVES

Studies on PKCθ since its discovery about 20 years ago have revealed an extensive amount of information about its expression, regulation and function, especially in T cells where it is expressed most abundantly. It is now clear that PKCθ plays important roles in T cell activation and survival by activating several downstream signaling pathways, with the NF-κB and

AP-1 signaling pathways representing major targets. The early characterization of *Prkcq*$^{-/-}$ mice, which was conducted in vitro, implied a global role in T cell activation, reflected by severe defects in TCR-induced activation, proliferation, and cytokine production. This notion raised some doubts regarding the utility and advantage of PKCθ as a drug target over other, widely used immunosuppressive drugs such as CN inhibitors, reflecting the concern that like, e.g., tacrolimus, it would globally inhibit immune responses, including protective responses against pathogens. However, later analyses by many groups of the ability of *Prkcq*$^{-/-}$ mice to mount various in vivo immune responses, including the use of experimental disease models, have led to the surprising, but clinically promising, conclusion that the requirement of PKCθ in the immune system is quite selective. These findings, combined with the predominant expression of PKCθ in T cells, make a strong case, at least from a theoretical standpoint, for its potential utility as a target for drugs that would display high selectivity and low toxicity. Indeed, progress in developing selective PKCθ inhibitors and early clinical trials have been reported, and there is clearly a sense that interest in this enzyme as a drug target for selective and beneficial immunosuppression is not waning but, rather, is on the upswing. Nevertheless, it is clear that there are still substantial gaps in our knowledge, which need to be explored and resolved before the full therapeutic potential of PKCθ-inhibiting strategies can be realized in the clinical arena.

1) Of prime importance among the unresolved questions is the importance of PKCθ in the *human* immune system. The overwhelming majority of PKCθ-related studies have been conducted in mice, and very little is known about the role and importance of this enzyme in human T cells. Interestingly, despite major progress over the past ~20 years in elucidating the molecular basis of many forms of human immunodeficiency, an immunodeficiency associated with impaired PKCθ expression or function has not yet been reported (to the best of our knowledge). Nevertheless, the limited amount of reports addressing the function of PKCθ in human T cells provides a basis for cautious optimism. First, early-stage small-molecule catalytic inhibitors of PKCθ inhibit the activation and proliferation of human T cells in vitro, subject to the caveat that these inhibitors likely inhibit other kinases in addition to PKCθ. Second, early clinical trials with one such inhibitor (AEB071), despite being inconclusive are encouraging. Third, the *Prkcq* gene has been tentatively identified as a potential risk factor in a few human autoimmune and inflammatory diseases. Fourth, and perhaps most relevant in this

regard, is the report that a selective small-molecule PKCθ inhibitor reversed the impaired suppressive activity of Tregs from RA patients (Zanin-Zhorov et al., 2010). These reports should serve as a strong impetus for exploring more extensively the importance of PKCθ in the human immune system. The in vitro use of various PKCθ inhibitors that are becoming available or RNAi-based knockdown strategies, as well as the expansion of clinical trials with PKCθ inhibitors, either alone or as components in combination with low, less-toxic doses of conventional immunosuppressive drugs such as tacrolimus could be very informative in this regard.

2) The seminal finding that PKCθ is excluded from the IS of Tregs and, more importantly, that PKCθ negatively regulates Treg-mediated suppression need to be extended in order to determine the mechanistic basis for its IS exclusion and negative regulation of Treg suppressive function. A possible explanation for the exclusion of PKCθ from the Treg IS was provided by Yokosuka et al. (2010), who showed that CTLA-4 competes with CD28 in recruitment to the cSMAC, thereby displacing the PKCθ–CD28 complex (Kong et al., 2011) from the IS. However, it is equally possible, at least theoretically, that PKCθ, perhaps in complex with some other partner(s), plays an active signaling role to inhibit the function of Tregs when it is localized in the distal T cell pole.

3) Although mouse *Prkcq*$^{-/-}$ T cells display severe activation defects, it is possible that effective immunosuppressive strategies based on PKCθ may have to take into consideration the need to inhibit other PKC family members that may play a compensatory role. This notion is supported by the reported cooperativity between PKCθ and other PKCs as described earlier, and the suggestion that the effectiveness of AEB071 in inhibiting T cell activation results from its ability to inhibit several other PKCs in addition to PKCθ (Evenou et al., 2009; Skvara et al., 2008). In this context, it is important to note that the use of pharmacological kinase inhibitors can result in functional outcomes quiet distinct from those observed in mice lacking that same kinase, emphasizing again the importance of conducting inhibitor studies in human T cells.

4) Despite the promise of early small-molecule catalytic PKCθ inhibitors, the use of similar kinase inhibitors in general is less than optimal because of their lack of absolute specificity, which often leads to toxic side effects. Therefore, development and exploration of allosteric inhibitors for PKCθ (and other PKCs that may participate in T cell activation)

is a worthy goal. Our recent study (Kong et al., 2011) demonstrates a new potential approach for attenuating PKCθ-dependent functions utilizing allosteric compounds based on the critical proline-rich motif in the V3 domain of PKCθ that will block its Lck-mediated association with CD28 and recruitment to the IS, an association obligatory for its downstream signaling functions. The pursuit of this new approach is worthwhile.

5) Last, it would be important to elucidate the mechanisms that render some types of immune responses, particularly T cell-mediated antiviral immunity, PKCθ-independent. In this regard, it has been demonstrated that inclusion of a TLR9 agonist in a T cell vaccine can rescue impaired T cell responses in *Prkcq*$^{-/-}$ mice, suggesting that certain TLR signaling pathway(s) can compensate for the lack of PKCθ (Marsland et al., 2007). One likely candidate is the NF-κB signaling pathway, which is a major PKCθ target in T cells, but is also activated by engaged TLRs. Therefore, it would be interesting to determine whether this compensatory activity of TLR ligands is shared by other TLRs.

These unresolved questions pave the way and provide directions for future high-priority studies that will improve our understanding of the role of PKCθ in the human immune system, and guide the development of what is likely to be a new generation of drugs that target PKCθ to induce desirable selective forms of immunosuppression. Such drugs may be able to eliminate or dampen deleterious immune responses such as autoimmunity and GvHD without impacting the ability of treated patients to eliminate harmful infections. In the coming years, we should see important and exciting advances along these lines and, hopefully, will realize the potential of PKCθ as a novel and highly useful drug target.

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ABBREVIATIONS

BMT Bone marrow transplantation
ChIP Chromatin immunoprecipitation
CN Calcineurin

CTL Cytotoxic T lymphocyte
GIST Gastrointestinal stromal tumor
GvHD Graft-*versus*-host disease
GvL Graft-*versus*-leukemia (response)
IS Immunological synapse
PI3K Phosphatidylinositol 3-kinase
PKC Protein kinase C
SMAC Supramolecular activation cluster
SNP Single nucleotide polymorphism
TCR T cell receptor
TLR Toll-like receptor
Teff Effector T cell
Treg Regulatory T cell
VSV Vesicular stomatitis virus
WASp Wiskott–Aldrich Syndrome protein

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