Davide Malagoli · Enzo Ottaviani
Editors

Eco-immunology

Evolutive Aspects and Future Perspectives

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ISBN 978-94-017-8711-6 ISBN 978-94-017-8712-3 (eBook)
DOI 10.1007/978-94-017-8712-3
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2014936184

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Immune system functions require energy. Energy availability is not indefinite and the immune system is not the only component requiring energy resources. As a consequence, the degree of energy allocated for the functioning of the immune system will depend on the total amount of available energy and the energy simultaneously required by other systems. The tight interconnection between energy availability and immune response is one of the main focuses of eco-immunology and it has been receiving an increasing attention in the last decade, with relevant implications for immunological studies. This book wants to give an "all-round" perspective of the possible approaches and ideas that may be included in, or may stem from, the eco-immunology.

Introducing the concept of energy allocation and redistribution, and describing the challenges presented by the on-field research, the first Chapter of this book poses one of the most relevant questions of the eco-immunologists. Is the lab-research a good and faithful representation of the diversity and the variety of immune challenges that an organism have to face daily in order to survive and reproduce? The answer is far from being obvious, and Chap. 2 makes the reader aware of the increasing complexity of the variables that must be considered by eco-immunologists, illustrating that population dynamics and mathematical models are ineludible instruments for investigating immune responses against infectious diseases. Thanks to this perspective, the interactions between host and pathogens may be analyzed and described using also ecological models leading in some cases to the unobvious conclusion that a less responsive immune system may in some cases improve the survival.

The cultural fundament of eco-immunology describing the immune response as a physiological activity that must be controlled in order to avoid an excessive energy consume is analyzed in depth throughout Chap. 3. The interactions between immunity and reproduction are analyzed, with a special attention towards the fundamental role played by trade-offs, a further concept representing a cornerstone of eco-immunology. Considerations on the Hamilton-Zuk hypothesis, an hypothesis formulated in 1982 and linking host-parasite dynamics with reproductive success, represent a trait d'union between Chaps. 3 and 5. In the latter, Owen and Hawley focused on host-parasite interactions, one aspect that, though fundamental, is frequently

overlooked in the eco-immunological studies aimed at understanding how ecological components intervene in shaping the immune system and its evolution.

The evolution of the immune system becomes the major topic in Chap. 5, where the concepts of trade-offs are extended to the immune-neuroendocrine system, here described as an integrated component both in terms of functions and evolvability. In Chap. 5 the principle differences between jawed vertebrates and other metazoans are analyzed in light of eco-immunological considerations. One of the principle elements of discontinuity observed in the immune systems of jawed vertebrates is represented by the lymphocyte, a cell with immune-neuroendocrine functions and the major component of the "mobile brain" described in 1986 by Blalock and Smith. The environment in which T lymphocytes mature is represented by the thymus, an organ that notoriously undergoes relevant changes during growth and aging. As evidenced in Chap. 6, thymus commonly hosts a great energy investment in childhood even in absence of important immune challenges, in order to provide a more efficient immune system in the adulthood. The redistribution of energy allocation implies programmed structural changes which, accordingly to Quaglino and coauthors, may not be finalized to the simple disposing of the thymus, but rather to its reutilization as energy storage and endocrine component. Chap. 6 also represents a transition point of the book, where evolutionary and ecological considerations derived from animal models give room to aspects of eco-immunology more connected with human immunology and translational medicine. The new and suggestive concept of "antigenic eco-space" is proposed in Chap. 7, in which the importance of the immunological biography of individuals is analyzed for its role in determining the quality of immuno-senescence. Of relevance, Franceschi and colleagues hypothesize that human genetic variants selected by evolution are probably unfit to the present-day environment of developed countries. mTOR- and NF-κB-mediated pathways are the mentioned examples of molecular routes that have been selected as adaptations to limited resources and whose action may reveal deleterious in aged people of wealthy countries. The alternative between immuno-senescence and the related state of chronic inflammation (inflammaging) versus an healthy aging is the main topic of Chap. 8. Interestingly, the role of geographical and cultural environment seems relevant in determining the prevalence of a detrimental inflammaging versus a positive one, and it is proposed the concept of "population immunology" to emphasize the importance of the environment in modeling the individual immune system and its functioning during post-reproductive age.

The reliability of laboratory models, the importance of mathematical analyses, the evolutionary response of adaptive immune systems to resource availability and the health problems suffered by humans in consequence of immuno-senescence are recollected in an integrated view within the Chap. 9. In this last Chapter, human and mouse immune systems are comparatively analyzed in the light of the directionality theory, a mathematical model of evolutionary dynamics. The evolutionary-driven differences between human and mouse immune systems, especially in their adaptive components, may have relevant consequences on immunological researches. More precisely, using Alzheimer's disease as principle reference, Chap. 9 introduces the discrimination between early- and late-onset diseases and it explains that

mice can be reliable models for the early-onset diseases, but they are unreliable guides for late-onset inflammatory diseases. As a consequence, therapies developed in mice and utilized for counteracting late-onset diseases will most probably be ineffective in humans.

Throughout the book it emerges the wideness of concepts that can be embraced by eco-immunology. Despite the different perspectives and expertise of the contributors, one of the most important leit-motif is the continuous and combined appeal to mathematical models, evolutionary theories and immunological competences. Much more than the combination of ecology and immunology, eco-immunology is progressively revealing itself as a self-standing discipline, a way of thinking and approaching numberless biological problems, which may have reflections on diverse fields extending from environmental and evolutionary analyses to therapeutic strategies and welfare politics.

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Chapter 1
From Immunology to Eco-Immunology: More than a New Name

Mark E. Viney and Eleanor M. Riley

Abstract Eco-immunology recognises that immune systems and responses are an integrated part of an animal's physiology, preventing harm that might be caused by pathogens. Laboratory-based immunology has given a tremendously strong understanding of the mechanisms by which this system functions, though this is focused on a few species. It is clear that immune responses are costly to make, such that animals have difficult decisions of resource allocation to make. For this reason alone, an eco-immunological approach is key to understanding the functional effect of immune responses. Animals live in antigenically complex environments. Because of the superb adaptiveness of the immune system (as well as its redundancy) the immune responses made by different individuals in a population may differ greatly, but the functional effect of this is unclear and ripe for investigation. The research challenges for eco-immunology are substantial, but achievable.

Keywords Costs of immunity · Immune regulation · Co-infection · Antigen diversity

1.1 Introduction

The immune system has been studied in great detail, for many decades, though largely in the laboratory. This has unpicked the many detailed mechanisms by which this complex system functions to produce immune responses. Eco-immunology seeks to understand the immune responses that are produced, and to what effect, in animals in their proper ecological context. Eco-immunology therefore views the immune system as an integral and dynamic part of how animals optimise their fitness in challenging, competitive environments. The detailed mechanistic understanding of immune systems has made clear that the functioning of immune systems is context-dependent. It is also clear that the environment in which most

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3_1,

laboratory-based immunology is undertaken is very different from that experienced by wild animals. From an ecological perspective, an animal's environment, and individual responses to it, are key to fitness. This, together with the clear context-dependent nature of immune systems, strongly suggests that immune systems need to be studied in wild animals. Here we consider key aspects of the biology of the immune system and immune responses especially relevant to eco-immunology; the contribution of immune systems to organismal fitness; and key challenges and questions both for eco-immunology and for laboratory-based immunology. Our focus is vertebrate-centric, though the principles apply across all organisms.

1.2 Why Have Immune Systems and Immune Responses Evolved?

All organisms survive by exploiting, to some extent, other organisms. Various degrees of intimate relationships exist between a host and other organisms, which range from symbiosis, through colonisation and commensalism, to parasitism and infection. For simplicity, we can think of pathogens as those organisms which cause harm to their hosts, recognising that this may be facultative and context-dependent. The world is full of pathogens seeking to make their living at the expense of hosts. Hosts are a rich resource that many pathogens wish to exploit. Without defence mechanisms, including the immune response, resource-rich hosts would soon be exploited to extinction.

1.3 What do Immune Responses do?

Immune responses have evolved to protect their owners from harm, especially harm caused by pathogens. The classic caricature of this is that an individual acquires, say, a virus infection. The viruses will multiply and if this is unchecked the host will be harmed or killed. The virus can cause this harm both because of direct effects (such as destruction of host cells) and indirect effects (such as the use of host energetic and metabolic resources). When the virus infection starts, the immune system begins to act. Both cellular and humoural responses may be made, which are both innate (thus general and common to any infection) and adaptive (that are highly specific to the infecting virus). In this caricature, the end result is that the immune response stops virus replication, the virus infection is cleared from the host, and the host is saved from harm and lives on without the possibility of reinfection. Even though this is a caricature, it is essentially true for many infections that animals (us included) acquire during our lives. The very fact that we and other animals have many infections during our lives shows that immune responses work; if they did not, a first infection would be fatal.

That said, this caricature is an over simplification. Many infections are not simply eliminated by an immune response; rather they are constrained and controlled to the point where they cause little, if any, harm to their host. The specific reasons for this depend on the infection in question. However, generally the "failure" of immune responses to eliminate an infection is due to an interaction between some limit of the possible immune response that is generated (because of (i) of active immune regulation, (ii) immune responses that cause harm to the host, and (iii) of other constraints, all discussed below) and the evolved responses of pathogens to the selective pressure that the host immune response imposes. The host immune response is an evolutionary selective pressure that affects how pathogens evolve. In essence, pathogen fitness is greatest if the immune response does not affect the pathogen (equally host fitness is greatest if pathogens do not affect hosts). Pathogens have therefore evolved ways to overcome or ameliorate the effects of host immune responses, to enhance their own fitness. Hosts and pathogens are therefore locked into an evolutionary/counter evolutionary "arms race". It is noteworthy that both animal (Davies et al. 2001; Viney and Lok 2007; Viney and Cable 2011) and plant (Bhattarai et al. 2008) pathogens use the host immune system as developmental cues. This shows how some parasites have evolved an exquisite inter-dependence on their hosts, above and beyond treating a host as a home and food source.

So far we have considered pathogens. However, not all infections are pathogens: some are always commensal, others are facultatively commensal or pathogenic. The challenge for immune responses is recognising and responding to pathogens, but not to commensals. The gut microbiota illustrates this well. The gut microbiota is a diverse assemblage of bacteria (>1000 species), in large quantities (about a kilogram of cells; 9 of every 10 cells in the human body are microbial), containing >200-times as many genes (*i.e.* the microbiome) as our own genome, with metabolic and other effects (Eckberg et al. 2005; Ley et al. 2006; Rajilic-Stojanovic et al. 2007; Turnbaugh et al. 2006, 2008, 2009; Candela et al. 2010; Ding et al. 2010; Pennisi 2010; Ley 2010; Diaz Heijtz et al. 2011). The majority of the gut microbiota are commensal; moreover, they are necessary inhabitants of the gut, without which normal development of the gut (Candela et al. 2010; Chung and Kasper 2010; Lee and Mazmanian 2010; Round and Mazanian 2009), normal physiological functioning, including immune function (Brugman and Nieuwenhuis 2010; Candela et al. 2010; Cerf-Benussan and Gaboriau-Routhiau 2010), and food processing would not occur. Clearly, immune responses against such commensal species would be detrimental to the host. Some species of the gut microbiota can be commensal or pathogenic, with this depending on their precise genotypes as well as their physical and microbial context. The immune system has evolved so that each host learns to not respond to obligate commensals (this is termed immunological tolerance), whilst retaining the potential to respond to newly arrived pathogenic species or species that have, facultatively, become pathogenic.

Here we have considered the pathogen (or commensal) as the driver (or limit) of the host immune response. However, the host also actively manages and limits its own immune responsiveness too. The key concept here is that immune responses can be costly, in at least two ways. Firstly, an immune response may not always be advantageous for

the host. Certain types of immune response can cause significant harm and damage to the host itself, as to the pathogen at which the immune response is aimed. Immune-mediated host damage is termed immunopathology and may, in some circumstances, cause more harm to the host than the infection which initiated the immune response. Therefore, immune responses have to strike a balance between maximising damage to the pathogens and minimising damage to the host (Graham et al. 2005). Immunopathology may be an unavoidable consequence of an immune response. For example, a virus inside a host cell may be best controlled by killing (lysing) the host cell; if this cell can be readily replaced (*e.g.* a gut epithelial cell) then the consequences of destroying the cell may be short-lived and cause only minor loss to the host. However, if this cell is terminally differentiated and difficult or impossible to replace (*e.g.* a neuron) then destroying large numbers of such cells may lead to permanent and severe host damage. Similarly, inflammation (*i.e.* a co-ordinated series of events leading to migration of immune cells to the site of infection, to clear the infection and repair host damage) can also cause host tissue damage. Inflammation can be accompanied by fever, pain and lethargy; fever helps to control infection (though fever is metabolically costly independently of immune responses, Baracos et al. 1987), pain and lethargy may be adaptive by limiting host activity which maximises host resources to fight infection and repair damage. In some cases the target of a pathological immune response may not be a pathogen, but an apparently normal host cell. Such auto-immune responses may however be triggered by infections which harm host cells, revealing damaged host molecules which then become the focus for an immune response which damages healthy tissue (Graham et al. 2005). Controlling immune responses to infection may thus also be required to prevent auto-immune diseases. Secondly, immune systems and immune responses require host resources—there's no such thing as a free immune response (see below).

When thinking about immune systems and immune responses, one needs to consider how natural selection acts on these, and the relationship between an immune system and its response. For example, when saying that "immune responses have to strike a balance" (see above) it is important to be clear that this "balance" is the evolved way in which the immune system responds, and the form of response in question is the result of natural selection. The immune system is a devolved system, in that each cell of the immune system has genomically-encoded rules of how to respond in any future infection environment. When an immune response is made, there is not a centralised control of function, but rather the immune response is the sum of the individual responses of each cell. Natural selection therefore sets these rules of function; these rules can be thought of as the quantitative relationship between stimulatory input and effector output. The immune system is therefore, in essence, the rules by which immune cells act and interact; the immune response is the combined output of these cells' action. There are two levels at which one can think of the evolution of the immune systems. The first is between-species comparisons, where differences in the structure and function of immune systems are the result of evolution. The second level is more interesting and relevant for eco-immunology, namely differences between individuals within a species. Here, while all individuals will have the same basic structure and functioning of the

immune system, the precise rules of how immune cells to respond in an infection environment will differ between individuals. The consequence is that there will be qualitative and quantities differences between individuals in the immune response that is generated in a common situation. In considering this, it is also clear that the immune system cannot be considered in physiological isolation.

In summary, while immune responses typically stop infectious agents exploiting hosts, this does not always happen because: pathogens have evolved to evade host immune responses; hosts have evolved to ignore commensal infections; and the costliness of immune responses limits what hosts do. Therefore, immune systems and responses have to be very carefully managed, controlled and optimised to provide sufficient net benefit to the host. This makes the point that immune systems should be seen as an integral part of an animal's wider physiological processes. The relevance to eco-immunology is understanding that animals have complex and different responses to different infections; the caricature of an infection always being stopped and removed by the immune response is too simplistic. Equally, understanding (or even predicting) what immune responses an infection will induce, and the consequent effect on the pathogen (if any, and, secondarily, any other effect on the host) is not always possible, especially for wild animals.

1.4 The Environment Affects the Immune System and its Responses

The immune system and its responses do not occur in a vacuum, rather they are part of the dynamic physiology of animals. How an immune system develops (to become a mature immune system, and to continue to adapt to an individual's lifetime of antigenic[1] exposure) and functions (varying qualitatively and quantitatively in the immune responses that are produced) is greatly affected by the hosts internal physiology, which itself is affected by the host's wider environment.

1.4.1 The Immune System Matures

The immune system of all animals matures with age and increasing antigenic exposure (*i.e.* by colonisation or by infection with commensal organisms or with pathogens) typically from commensal and/or pathogenic organisms. Maturational changes of the immune system include production of specific antibodies, an increase in the number of cells of a particular population (*via* cell division; clonal expansion is a typical response of an immune cell to activation by antigen) and functional

[1] An antigen is a molecule to which an immune response is induced. Antigens are typically from pathogens, although experimentally any molecule introduced to an animal acts as an antigen. Auto-antigens are host-derived molecules to which, therefore, an auto-immune response is generated.

maturation of those cells as a result of physical, molecular encounters with pathogens. Therefore, the precise characteristics of the immune response of an individual will be determined, in part, by their age (*e.g.* Nussey et al. 2011 in wild animals) and their precise prior antigenic experience, including its temporal sequence. In addition, the immune system has evolved numerous and diverse pathways by which it acts. The immune system has in-built redundancy, such that if one component of the immune system is defective or disabled (*e.g.* by mutation or by immune-regulation), other components are able to compensate. This redundancy underlines the essential contribution of the immune system to fitness but it also means that different individuals may achieve the same or similar outcome of antigenic challenge by quite different immunological routes. This may be manifest as inter-individual differences in the immune responses that individual animals (of similar ages, in the same environment) make against the same antigen or pathogen.

1.4.2 Immune Responses Cost

Once an immune system has matured, the quantitative and qualitative nature of the immune responses produced are still affected by the host environment. A key concept here is that immune responses have an energetic cost, and investment of energy in immune responses has to be balanced by the use of energy for other aspects of life. Here it is assumed that an animal has a limited energy budget, so choices about allocation of energy have to be made. The evidence in support of these concepts is compelling, in three ways (Lochmiller and Deerenberg 2000). Firstly, direct measurement of the energy cost of mounting an immune response has been made in laboratory animals. For example, mice making anti-keyhole limpet haemocyanain (KLH) IgG responses have an increased oxygen consumption, compared with control animals (Demas et al. 1997). Collared doves (*Streptopelia decaocto*) making an anti-sheep red blood cell (SRBC) immune response also increase their basal metabolic rate, compared with control animals (Eraud et al. 2005). In sparrows (*Passer domesticus*) anti-phytohaemagglutinin responses use *c.*4.2 kJ/day, which is equivalent to the cost of production of half an egg in this species (Martin et al. 2003). Measurement of the energetic cost of nematode (*Trichinella spiralis*) infection, (compared to SRBC administration or control animals) found that the nematode infection had a measurable metabolic cost (mass-controlled oxygen consumption) whereas the SRBC-administration did not (Martinez et al. 2004). On the other hand, the energetic cost of remaining healthy in the absence of a fully functional immune system can be higher than the cost of maintaining the immune system. Thus, lymphocyte-deficient mice have a higher basal metabolic rate than the normal mice, suggesting that the compensatory up-regulation of the innate immune system (required to off-set the lack of an adaptive immune response) has a greater energetic cost than maintaining a normal combination of adaptive and innate immunity (Råberg et al. 2002).

Secondly, wild animals have been administered foreign antigen, and the effects on other aspects of their biology (especially those that are themselves costly)

observed. Alternatively, animals' "workloads" have been altered and the effects on immune responses observed. Many of these studies have been done on nesting birds (which makes them accessible), but these animals may already be energetically stressed because of reproduction and thus these results may not be generalisable to non-reproducing individuals. Notwithstanding, these studies have shown that antigen administration does alter aspects of the biology and behaviour. For example, Eider ducks (*Somateria mollissima*) which mounted a humoral immune response after injection with foreign antigens (SRBC and diphtheria-tetanus vaccine) had reduced migration return rates compared with those that did not make such an immune response (Hanssen et al. 2004). Another study with wild blue tits (*Parus caeruleus*) investigated how immune responses to vaccination were related to over-winter survival, a measure of fitness (Råberg and Steinman 2003). This found evidence of stabilising selection on a primary immune response and of directional selection on a secondary immune responses; taken together this suggests that there is a balance between the fitness advantage of immune responses and the cost of making them (Råberg and Steinman 2003). House sparrows (*Passer domesticus*) injected with lipopolysaccharide (LPS) have decreased activity, body mass, feeding rate and reproductive success, compared with non-LPS administered controls (Bonneaud et al. 2003). Conversely, if the workload of birds is increased, immune responses are diminished (Deerenberg et al. 1997; Ewenson et al. 2003). For example, in tree swallows (*Tachycineta bicolor*), increasing brood size reduces secondary antibody responses to SRBC administration (Ardia et al. 2003) and clipping of flight feathers lowers antibody responses to keyhole limpet haemocyanin (KLH) (Hasselquist et al. 2001). Various immunological responses, as well as next-year reproduction, are lower in Eider ducks given higher egg incubation loads (Hanseen et al. 2004).

In all these studies, the effects of antigen administration are assumed to be because the induction of a costly immune response is paid for by reduction of other costly activities and behaviors. This supports the idea that animals have a limited available energy budget and that there is competition between different energy-costly activities. However, some caution does need to be applied to these studies because (i) there may be a publication bias towards positive results (Williams et al. 1999) and (ii) many of these effects have not been formally demonstrated to be immune-dependent (Martin et al. 2003; Viney et al. 2005). Notwithstanding, in these studies, the currency of the cost can be thought of as energy (*i.e.* kcal) but this is often not known, nor is it particularly key to the wider concept. The currency could be energy expenditure *per se*, the protein content of the diet, quantities of micronutrients (*e.g.* Koski and Scott 2001), or it could be a deficit in energy intake as a result of feeling "unwell" or "unmotivated" (*e.g.* due to the fever and lethargy associated with inflammation) (Lochmiller and Deerenberg 2000). Whatever the currencies are (and there could be multiple currencies acting together) as long as they translate making an immune response into a reduction in some other activity, then this shows the cost of an immune response. As far as we are aware the induction of an immune response has not been shown to be accompanied by an *increase* in some aspect of activity or behaviour.

The third approach to studying the cost of immune responses is to modify the energy *etc*. intake of animals and then to measure the effect on immune responses. Overall, these studies have shown that a reduction in energy intake alters immune responses. For example, mice infected with the nematode *Heligmosomoides polygyrus* and fed mild energy (but not protein) restricted diets had suppressed measures of immune function including cytokines (interferon gamma (IFNγ), interleukin 4 (IL4), IL5), antibodies (IgG_1) and cells (eosinophils) in the gut and spleen compared with normally-fed controls (Koski et al. 1999). Analogous effects were detected with protein-restricted diets (Ing et al. 2000). A further point that emerges from these many studies is that different changes in energy, protein, or micronutrient content of the diet can have different immunological effects (Koski and Scott 2001). Low protein diets can shorten immune responses (Jose and Good 1973); calorie restricted diets can reduce delayed type hypersensitivity (DTH) responses (Pocino et al. 1987). Among sheep maintained on different nutritional planes and vaccinated against the nematode *Trichostrongylus colubriformis*, those on the higher nutritional plane responded better (both immunologically and functionally) to a challenge infection of *T. colubriformis* (Wagland et al. 1984; also see Koski and Scott 2001). Again, these studies support the concept that immune responses require energy and that when energy is limited, measures of immune responses (and, indeed, immunity itself) are reduced.

Analogous "natural experiments" in people also confirm that a reduced diet alters immune responses. Here, immune responses (for example to vaccination) have been measured among individuals in various states of malnourishment. Vaccination of malnourished children can impair their weight gain (Kielmann 1977). Among malnourished and normally nourished children, most measures of immune function (especially of cellular immune function) were positively correlated with measures of nutritional status, and supplemental feeding rapidly restored immune function (Neumann et al. 1975, 1977), indicating that the immune system has the capacity to respond very quickly to changes in the availability of energy and other resources. A study of growth in height and a measure of the immune response (specifically C-reactive protein (CRP) concentrations) in rural Bolivian children found that greater CRP concentrations predict lower height gains (McDade et al. 2008). Here, the idea is that investment in immune responses (*i.e.* CRP) is paid for by less investment in growth. From the eco-immunology perspective, it is noteworthy that anthropological studies are considering how human immune responses affect human life-history, especially with the view that early-life infections and immune responses that are required for defence and repair use substantial energy that is allocated at the expense of growth (McDade 2003; Crimmins and Finch 2006).

The results of these animal and human studies are sometimes counterintuitive. Intuition suggests that a reduced energy status will result in reduced measures of immune responses, but empirical observations do not suggest such a simple relationship. While it is clear that reduced energy status alters measures of immune responses, not all types of responses are reduced (Good et al. 1977). It does, though, strongly emphasises that immune responses dynamically vary depending on the energy status of the individual

In summary, it is clear that the environment affects the development and continued maturation of the immune system and, moreover, that the magnitude and the nature of immune responses an individual makes is dynamic and is affected by the environment. This is most clearly understood with the perspective that energy-costly immune responses require allocation of resources away from other energy-costly aspects of life. This has clear relevance for eco-immunology: if environment is, immunologically, everything, then the functioning of immune systems can only be properly understood by considering it in a natural environment, in which it evolved. It is abundantly clear from laboratory studies that the very special environment experienced by laboratory animals profoundly affects their immune system and its function. At the moment the extent of the disconnection between immune functions measured in the laboratory and immune functions measured in wild animals is really very poorly understood or appreciated. It is key that this deficit is addressed rapidly.

1.4.3 The Immune System has Multiple Antigenic Challenges

In the caricature of a classic immune response against a virus infection (1.3 above), the immune system was dealing with a single infection. The real world is more complex. Immune systems exist and have evolved in the face of continued, multiple (and often concurrent) infections. As we have already discussed, the first challenge for the immune system is to make an appropriate (energetically affordable, effective at containing or removing the pathogen, and non-harmful to the host) response to an infection. The challenge is actually greater still, because the immune system needs to make many appropriate responses to different infections. Studies of co-infection in recent years have shown that co-infections do matter. Specifically, if two infections occur together in a host these infections differ (both by measures of infection *per se* and of host-pathology) compared with when each infection occurs individually. These co-infection effects can be either positive for the host (infection A ameliorates infection B) or negative (infection A enhances infection B), and can be unidirectional (A affects B) or reciprocal (A affects B and B affects A). These effects have been shown both by controlled laboratory studies and by epidemiological surveys of humans and other animals. Co-infection effects and their consequences should come as no surprise; in free-living ecosystems inter-specific interactions frequently determine species' abundance. Studies in wild and laboratory animals have shown just how strong these co-infection effects can be (Lello et al. 2004; Graham 2008; Segura et al. 2009; Telfer et al. 2010). For example, in wild voles four pathogens (one virus species, one protozoan species, and two bacterial species) were measured over time and co-infection was found to be more important than season or host age in determining susceptibility to these infections (Telfer et al. 2010). The mechanisms underlying these co-infection effects have not been fully worked out. Effects could occur directly (by one infection producing molecules that affect other infections) or indirectly (by competition for a limiting host resource). Equally, co-infection effects can be (and have been shown

to be) immunologically-based (Graham 2008). For example, antibodies induced to control infection A may also affect infection B if (i) infection B has cross-reactive antigens or (ii) the immune response induced by infection A reduces or alters the immune responses against infection B due to immune regulation or immune modulation. For example, infection of mice with malaria inhibits the oxidative burst of neutrophils (which are an anti-microbial response) which reduces the immunopathology of the malaria infection. This is enormously beneficial to a mouse if it is only infected with malaria (and allows the mouse to survive the infection). However, this effect is enormously detrimental to a mouse if it is infected with malaria and *Salmonella* because the consequent absence of the oxidative burst leaves the *Salmonella* infection un-checked (*i.e.* the mouse anti-*Salmonella* resistance is lost) and the mouse succumbs to salmonellosis (Cunnington et al. 2012).

The importance of multiple antigenic challenges, including co-infection, for eco-immunology is obvious. Laboratory animals are kept in such a way so as to minimise the infections to which they are exposed. Laboratory-based studies of infections typically only consider one infection at a time (although this is changing). In stark contrast, wild animals will be continually exposed to a wide range of infections—co-infection will be the norm. This means that the immune system of wild animals will be functioning in an environment in which it evolved and therefore one in which it is appropriate to study. Equally, this means that the immune systems of laboratory mice are being studied in environments far removed from those in which they evolved, and thus that observations from laboratory studies cannot be expected to translate easily or reliably to ecological studies. Moreover, it is very clear from studies of laboratory animals that, apparently, subtle differences in the dose, timing and site of antigen administration can very significantly affect the immune response that develops (which, further, varies through time) (Paterson and Viney 2002; Bleay et al. 2007). These studies show the importance of an eco-immunological approach, but also the experimental and intellectual challenge.

Immune responses are further constrained because an immune response of one type may (necessarily, in the case of immune regulation) counter-regulate an immune response of another type. The original example of this is the relationship between adaptive so-called type 1 T helper cell (Th1) responses (typically directed against intracellular infections) and type 2 (Th2) responses (typically directed against extracellular pathogens, particularly parasitic worms). Since the immune responses mediated by Th1 cells antagonise the responses mediated by Th2 cells (and *vice versa*), Th1 responses actively suppress Th2 responses, and *vice versa*. In addition, T cells whose only role is to regulate all other responses (so-called Treg cells) regulate both Th1 and Th2 cells (and several other recently defined T cell populations). These are examples of how immune regulation limits which immune responses can be produced. In fact, within the immune system, immune regulation that limits possible immune function is likely to be the norm and is essential for maintaining immune homeostasis or allostasis (Schulkin 2003; Xiao et al. 2011).

Such immunoregulation is an evolved feature of the immune system, presumably to optimise fitness given the antigenic challenge of the environment in which

it evolved. Thus, if the environment changes, then the immune system and its response may no longer be optimal for fitness. This is well illustrated by the hygiene hypothesis. The context of this is that humans (and our immune systems) evolved when there was continued and sustained antigenic challenge. In recent decades, in rich industrialised societies, people have lived in cleaner environments, such that individuals have overall less antigenic challenge to their immune systems. The result of this is that fewer Th1 and Th2 immune responses have been induced, and thus fewer Treg responses have been required to regulate them. It is hypothesised (Maizels 2005) that the consequent lack of Treg capacity may explain the (apparent) inexorable rise in the prevalence of allergic and auto-immune diseases in these societies.

In summary, eco-immunological studies need to consider that the immune responses of individuals will be driven by a complex interaction of factors most of which will be unknown (especially from single time point, cross-sectional samplings of a population). These factors include prior exposure to any particular antigen or pathogen, prior exposure to similar (cross-reactive) antigens, prior exposure to pathogens that induce counter-regulatory responses (be they Th1, Th2 or Treg, or others) and by the changing dynamics of the pattern of exposure to antigens and pathogens over time.

1.5 Immune Responses, Fitness and Health

The immune system and its responses have evolved to contribute to maximising fitness. *A priori*, as in our initial caricature of an immune response, fitness might be thought to be optimised by the immune system generating an immune response that quickly clears the infection in question. As the preceding sections have shown, this is not necessarily the case. Quickly clearing an infection may not be optimal because: (i) this would affect responses to other co-infections which, ultimately, would be worse for the host, and (ii) the costly resources required to achieve this (and/or the immunopathology incurred) would, ultimately, be worse for the host. Therefore, apparently sub-maximal (the infection is cleared only slowly), partially effective (the pathogen persists, but at a very low level) or non-effective (the infection persists and/or replicates in an unconstrained way) immune responses may actually be optimal for maximal fitness (Viney et al. 2005). It follows, therefore, that measures of host health and host pathology (concepts which, typically, come from human and animal medicine) are not obviously correlated with fitness. This does, though, lead to a conundrum: while apparently sub-maximal and non-effective immune responses may maximise fitness, the same sub-maximal and non-effective immune responses may be just that, and may not be contributing to fitness. A key challenge, therefore, for both laboratory immunology and for eco-immunology is to seek to understand how observed immune responses contribute to fitness (while recognising that fitness isn't necessarily good health). Some studies have measured

a component of fitness (*i.e.* reproductive success (Bonneaud et al. 2003), return rates (Hanssen et al. 2004), overwinter survival (Råberg and Steersman 2003)) and shown relationships (sometimes complex) with immune responses. However, it is clear that much more work remains to be done here.

In eco-immunology it is also important to consider individual animals. Specifically, any two individuals in the, apparently, same environment will face different antigenic challenges, and will also have different amounts of energy available, as well as different competing demands on those resources. Thus, each animal will have its own microenvironment of antigenic challenge, energy resourcing *etc.* Consequently, different individuals in the same ecological environment may be using their immune system in different ways, making different immune responses, but each individual optimising their fitness in so doing. For eco-immunology understanding the extent of this inter-individual variation and how such differences all contribute to fitness is a key challenge. Comparison of immune responses of wild-caught and laboratory-bred animals has found comparatively greater variation between the wild-caught animals (Devalapalli et al. 2006; Abolins et al. 2011; Boysen et al. 2011). Laboratory-based immunology has, essentially, sought to exclude differences between individuals by minimising environmental and genetic heterogeneity. The decades of laboratory-based immunology are, then, almost the study of one genotype of mouse in one environment.

This idea that individuals may differ in the "immune strategy" that they pursue can be extended to considering differences between species. Eco-immunology is relevant to different species (just considering the vertebrate immune system, then all species of vertebrates), whereas laboratory-based immunology principally studies the mouse (with some work on other rodents and on domesticated species such as sheep, pigs, cattle and chickens). The life-histories of different species vary enormously. Consider a mouse and an elephant. A mouse will probably live for less than a year, will aim to reproduce several times during its life, all within a short-term, intensely competitive environment in which stochastic events may be powerful. An elephant can live 50 years or more, may reproduce slowly over several decades, but will also have a long post-reproductive life. These two species may have very different approaches of deploying the resources available to them in immune function, as well as to other aspects of life and components of fitness (Westerterp 1978; Speakman 1997; Lochmiller and Deerenberg 2000). The mouse lives a short, intense life, in which acute, high-cost infections may have the greatest effect on lifetime reproduction, so that immune protection against such infections maybe the most profitable strategy. In contrast, for the elephant, an acute, high-cost infection, while undesirable, may have rather little effect on lifetime reproductive success. Instead, for elephants, mounting effective immune responses against chronic infections may have greater long-term benefit. There are rather few data relevant to these type of considerations (though see Nunn et al. 2000; Read and Allen 2000). However, an interesting example concerns two life-history ecotypes of the garter snake (*Thamnophis elegans*); one ecotype has a fast pace of life, the other slow (age at maturity 3 and 5–7; median lifespan 4 and 8 years, respectively) (Sparkman and Palacios 2009). Here the fast-paced ecotype has higher measures of constitutive innate immunity,

compared with the slow-paced ecotype, (Sparkman and Palacios 2009) and these differences are, at least in part, genetically controlled (Palacios et al. 2010). More broadly, however, a clear warning for eco-immunology is that different species may use their immune systems in very different ways, to achieve the same evolutionary ends. Eco-immunology therefore has the opportunity to show how what is known from well studied laboratory species (mouse, chicken *etc.)* might, or might not, be true among a greater species range.

1.6 The Challenge for Eco-Immunology

1.6.1 The Functional Effect of Immune Responses

As stated at the outset, eco-immunology seeks to understand what immune responses, and with what effect, occur in wild animals and how this contributes to their fitness. Measuring immune responses can be very easy (though see below). For commonly studied species (human, mouse, rat, chicken) very many immunological tools are available, so that a myriad of measurements can be made. Despite this ease of immune measurement, understanding the functional effect *per se* (let alone as a component of fitness) of these responses is far from clear; this is far from clear in a laboratory setting, and therefore even more difficult in natural environments. Many of the parameters of immune function used in eco-immunology (spleen size, total immunoglobulin concentration, responsiveness to a model antigen such as KLH or SRBC, or to a generic stimulant such as phytohaemagglutinin) give no real indication of the capacity of an animal to develop an optimal immune response to an infection. In other words, as discussed above, it is evident that altering energy budgets or workloads of animals can affect measures of their immune responses, but this gives almost no understanding of the fitness consequences of these changes; there may be none, or they may be profound. Therefore a challenge for eco-immunology may be to make fewer measurements of immune function, but to have greater understanding of their functional effect and the relevance to fitness (though this clearly not a straight-forward problem). One way in which laboratory immunology may contribute to this is by helping to identify the immune parameters that are most relevant for the specific infections, or combinations of infections, that are believed to compromise fitness in individual wild populations.

For most wild species, however, there are only very limited, or even no, immunological tools available. Therefore "traditional" immunological tools need to be adapted to these species or new species-specific tools need to be developed. Particularly attractive are assays that use nucleic acid sequence information (which is now so readily available), such as quantification of relevant gene transcription (*e.g.* Chaussabel et al. 2010; Jackson et al. 2011). Beyond developing appropriate assays of functionally relevant immune responses, the long standing difficulty of estimating fitness in wild populations continues.

1.6.2 Epidemiology and Ecology vs. Controlled Laboratory Experiments

The style of study of eco-immunology is also very different from laboratory-based immunology. Laboratory-based immunology typically conducts replicated, controlled experiments. This has allowed the detailed mechanistic function of the immune system to be understood. In contrast, for eco-immunology (as for any study of a wild population) cross-sectional or longitudinal studies need to be undertaken. In many ways this is very similar to epidemiological studies of human populations. Such studies collect data from quite large numbers of individuals on single (cross-sectional) or multiple (longitudinal) occasions and, statistically, seek relationships between, in our case, immune measures, and relevant aspects of host biology. In such studies noise and variance are key, which is typically anathema to laboratory-based immunology. As mentioned above, it is clear that, apparently, subtle differences in antigen dose, timing and site of administration of an antigen can significantly affect the immune response that develops (which, further, varies through time). Therefore, this further emphasises (as 1.6.1) the desirability of having fewer measures of immune responses, but selecting those are robust in measuring and capturing a functional effect.

This epidemiological-style of study will detect patterns of association that are common across populations (though proving causality of inferred effects is harder and typically requires a targeted intervention to change parameters and observation of the consequences). However, epidemiological studies also allow analysis of inter-individual differences in immune responses which, as discussed above, may occur among wild animals. Consideration of inter-individual differences in immune function will also require that the genetic contribution to immune responses is studied (Turner et al. 2011). This highlights another area where eco-immunology can develop an understanding of how immune responses contribute to fitness. Methods exist to assay the extent of selection acting across the genome, which means that the degree of selection acting on genes whose products have a key immunological role can be measured. At the moment this can only be done for genes that are sufficiently well characterised (often by homology with immunologically well-studied species such as the mouse) genes. However, genome-wide association studies (GWAS) can, in principle, also be used to identify the genetic basis of immune phenotypes that vary in a population, which might be more appropriate for non-model species than using candidate gene approaches. Notwithstanding which approach is used, the bigger difficulty is in understanding the immunological functional effect of the locus or loci in question; this is analogous to the difficulty of relating measures of immune responses to the functional effect of those immune responses.

1.6.3 The Antigen and Energy Microenvironment

Despite knowing a lot about immune systems, we know very little about the antigenic challenges a wild animal faces during its life. For certain classes of

infection (arthropod ectoparasites, metazoan and protozoan endoparasites, and some well characterised bacterial and viral infections) these can be readily diagnosed in wild animals and so these infectious challenges can be determined. In many cases the same, or similar, infections will also have been studied in laboratory animals. Beyond this, though, it is clear that there is a vast range of other bacteria and viruses (and very many, completely unknown and uncharacterised), as well as other antigens, to which animals are exposed during their lives. Beyond not knowing the diversity of the antigenic challenge of animals, a further unknown is the relative size of the antigenic challenge of these different infections. The size of these challenges may not be directly related to the size of the infecting organism. (For example nematode endoparasites seem to be antigenically rather small for their large body size). We do not know whether wild animals face a continuous high-level exposure to antigen (to which the immune response is always responding, so that animals are "running to stand still") or whether significant antigenic challenges are episodic in nature. One approach to investigating this may be to use methods that capture the peptides (processed from the stimulating pathogen) within antigen presenting cells (*e.g.* Phelps et al. 1996; Suri et al. 2003). With the abundance of genome information available, making links between this peptide diversity and the antigenic source may be tractable, although this would only ever capture the current and very recent antigenic exposure. Another possible way to consider studying this is to use measures of T cell diversity (perhaps sequentially through the lives of wild animals), with the assumption that the extent of T cell diversity is positively related to the degree of antigenic exposure; this has the added advantage of being able to capture historical as well as current exposure.

Analogously, while the importance of energy budgets in affecting immune responses is clear, we also have rather little understanding of the energy budget of wild animals. Fat deposits are usually observed in wild animals, suggesting that there is an excess of energy available. However, it is equally clear that immune responses are dynamic and responsive to short-term changes in energy input. The resolution of this possible paradox may be that the immune responses rely-on readily available (not deeply stored) energy whereas other costly processes such as reproduction use more deeply-stored energy. Small animals can eat food equivalent to half, or more, of their own body weight daily (Hawkins and Jewell 1962) and fat reserves may only provide a few days of total energy requirements, and that energy expenditure is dynamic (Westerterp 1978; Speakman 1997; Mclean and Speakman 1999). This further suggests that the cost of immune responses really do matter. It is clear that a better understanding of the energy budgets and dynamics of wild animals would be interesting.

1.7 Conclusion

The next necessary leap forward in immuno biology needs to be eco-immunology. Laboratory-based immunology has given a tremendously strong base for this endeavour, specifically a conceptual framework for thinking about organismal

responses to infection and protection from harm and, for some species, tools and means of analysis. Eco-immunology's big challenge is to consider the functional effect of immune responses in the wild. This is difficult, but one that if successful will feed-back to laboratory-based immunology, which could therefore link detailed mechanistic understanding of immune function with how immune systems and their responses contribute to fitness. It has famously been said that nothing in biology makes sense except in the light of evolution (Dobzhansky 1973); perhaps we will find that nothing makes immunological sense except in the light of the wild.

Acknowledgements Mark Viney would like to thank the Wissenschaftskolleg zu Berlin for a fellowship which enhanced this work.

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Chapter 2
System Biology Models and Conceptualizations Applied to Eco-Immunology

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Abstract This article outlines how ecological concepts, based on population dynamics and mathematical models, can be helpful for investigating various aspects of immunology, and in particular immune responses against infectious diseases. The review starts by describing how basic ecological concepts have been applied to investigating the *in vivo* dynamics between immune responses in viral infections *in vivo* and discusses the usefulness and limitations of such approaches. These concepts include predator-prey dynamics, competition between different clones of the same immune response, and competition between different branches of the adaptive immune system. The article continues to discuss how immune responses can be important modulators of pathogen competition *in vivo*, and this is explored with a particular example, i.e. the competition dynamics among different viral strains in the context of multiple infection of cells. Finally, these *in vivo* considerations are extended to examine how immune responses to infectious diseases can influence pathogen-host dynamics on an epidemiological levels. This is done with two examples: the effect of immunity on the apparent competition between two host species, and the effect of immunological memory on the strain composition of a pathogen population circulating in a host population. Overall, this article summarizes different aspects in which ecological concepts can be useful to understand concepts in immunology, discussing several different examples, spanning a variety of scenarios.

Keywords Coinfection · Host-pathogen dynamics · Virus competition

2.1 Introduction

The immune system is incredibly complex and involves the interactions of many different components ranging from molecules to cells to multi-cellular pathogens (Janeway et al. 2005). For a comprehensive understanding of the immune

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3_2,

system, such interactions have to be considered on a number of different levels or scales. Interactions of the different components *in vivo* are the most common considerations. In this respect, complex intracellular interactions determine the behavior of immune cells, such as their activation status, differentiation, and their activity. On a higher level, the interactions between different immune cell types, cytokines, and pathogen populations are essential for understanding the outcome of infections and for understanding the conditions for successful pathogen control or for failure of the immune system to contain pathogens (Nowak and May 2000; Perelson 2002; Wodarz 2006). In addition to these *in vivo* considerations, however, larger scale ecological and epidemiological interactions can have important implications for our understanding of immunity as a whole, especially when investigating the immune system from an evolutionary point of view (Thomas et al. 2009).

At all of these levels, systems biology approaches have been of great value for our understanding of these complex interactions. While verbal and graphical reasoning cannot provide a full understanding of the consequences of these interactions, mathematical and computational approaches allow us to formulate a certain set of biological assumptions in terms of equations or algorithms, and to follow them to their precise logical conclusions. This can add essential information to the work derived from experimental approaches. Much of this work has strong roots in the field of ecology, in particular population dynamics. The interactions among the different molecules, cells, and pathogens can be viewed as an *in vivo* ecosystem where elements compete with each other or act in predator-prey like fashions. The review will start with a detailed discussion of this concept. This will be followed by a case study that shows how immune responses can be important for determining the outcome of interactions and for shaping the evolutionary dynamics of pathogens *in vivo*. The second part of the review will consider the immune system and immune-mediated pathogen control in a broader ecological and epidemiological setting. Rather than concentrating on the in "*in vivo* ecosystem", it will highlight how ecological interactions among hosts, as well as between host dynamics of pathogens, can shape our understanding of the role of immunity in the light of evolution.

The immune system contains several different branches, and different pathogens elicit different responses. In order to keep this review focused, the adaptive immune system will be considered in the context of viral infections. The adaptive immune system includes cells that specifically recognize pathogens and that are essential for their control, such as killer T cell responses (also called CD8 T cell or cytotoxic T lymphocytes, CTL), helper T cell responses (CD4 T helper cells), and B cell responses (leading to antibody production). The innate immune response, which provides a first line of defense and which also collaborates with the adaptive immune responses, will not be considered. Even within this restriction a vast literature exists that examines these interactions from a systems biology point of view. Hence, the review will seek a balance between explaining general concepts and providing examples that are based on my own studies.

2.2 Immune-Pathogen Interactions as *in vivo* Ecological Systems

While immunology has traditionally been considered in the context of molecular and cell biology, it represents a highly complex system with many interacting populations of cells and molecules, that in turn interact with growing pathogen populations. Hence, a variety of systems biology approaches have been very useful for gaining insights into these complex interactions. A lot of this work is routed in ecological principles, particularly in the field of population biology, and mathematical approaches have played important roles in this respect. To this day, much of the theoretical work that considers the dynamics between immune cells and infectious agents is based on the concepts of predator-prey as well as competitive interactions. In order to demonstrate this point, let us consider one of the most basic models that describe the dynamics between a virus population and an anti-viral immune response (Nowak and May 2000). This model contains four variables: the populations of uninfected cells, S, infected cells, I, free virus, V, and a specific immune response, C. These quantities can either denote the total abundance in a host, or the abundance in a given volume blood or tissue. It is given by the following set of ordinary differential equations:

$$\begin{aligned} \dot{S} &= \lambda - dS - \beta SV \\ \dot{I} &= \beta SV - aI - pIC \\ \dot{V} &= kI - uV \\ \dot{C} &= \eta IC - bC \end{aligned} \tag{2.1}$$

Uninfected cells are produced with a rate λ, die with a rate d, and become infected by free virus particles with a rate β. Infected cells die with a rate a and produce new virus particles with a rate k. Free virus particles die with a rate u. An important concept in this respect is the basic reproductive ratio of the virus, R_0. This measure expresses the average number of newly infected cells produced by a single infected cell at the beginning of the infection, when almost all cells are uninfected. This measure determines whether a virus can successfully establish an infection in the host or not. If, $R_0 > 1$, then one infected cell on average gives rise to more than one newly infected cell, and the virus can successfully establish an infection. On the other hand, if $R_0 < 1$, then one infected cell on average gives rise to less than one newly infected cell, and the virus population goes extinct. On top of these basic virus dynamics, a virus-specific immune response is included in the model. In particular, this model considers a cytotoxic T lymphocyte (CTL) response, which expands in response to antigenic stimulation and kills infected cells through lysis. The CTL proliferate in response to antigenic stimulation with a rata η, and decay in the absence of stimulation with a rate b. CTL can kill infected cells with a rate p.

In this basic model, the equation for the CTL response is based on the Lotka-Volterra model for predator-prey interactions. The CTL are the predators that

proliferate in response to their prey (virus) and kill the prey. Consequently, the model has properties that are similar to those of the Lotka-Volterra class of models. If the CTL do not expand fast enough in response to antigenic stimulation, the response cannot become established and the following equilibrium is attained.

$$S^* = \frac{au}{\beta k}; I^* = \frac{\lambda\beta k - dau}{a\beta k}; V^* = \frac{\lambda\beta k - dau}{a\beta u}; C^* = 0.$$

If the CTL do expand sufficiently fast (i.e. if $\eta I^* > b$), then the population of cells grows and starts fighting the virus population. This can lead to oscillatory dynamics that will eventually dampen down towards a stable equilibrium. A typical time-series that arises from this model is shown in Fig. 2.1. The equilibrium is given by the following expressions.

$$S^* = \frac{\lambda u\eta}{\eta ud + \beta kb}; I^* = \frac{b}{\eta}; V^* = \frac{kb}{\eta u}; C^* = \frac{\eta\lambda\beta k - \eta dau - ba\beta k}{p\left(\eta ud + \beta kb\right)}$$

In this model, the equilibrium virus load is determined by the immunological parameters η and b. The faster the rate of antigen-induced CTL expansion (higher η) and the slower the death rate of CTL (lower b), the lower the number of infected cells, and thus amount of free virus. This model is deterministic in nature, that is the immune response cannot lead to the extinction of the pathogen. However, if equilibrium virus load is low or if oscillations before equilibrium is reached bring the number of infected cells to low numbers (of the order of one), this can be interpreted as immune-mediated pathogen clearance, since this would occur in a stochastic version of this model. If the immune response is not strong enough, the number of infected cells remains at significant numbers and a persistent infection is established, characterized by the equilibrium described above.

While this basic framework is based on predator-prey dynamics, it has to be noted that it is a very simplified representation of how immune responses, and CTL responses in particular, work. Many models have been developed that are based on this simple framework, but include many other experimentally established findings in the assumptions. Before the host is infected with a given pathogen, specific CTL are present in a "naïve" state, that is they are resting and found only at low numbers. Upon infection, the pathogen activates these naïve CTL, and the activated CTL undergo clonal expansion that eventually leads to the differentiation into effector cells, which are the cells that actually fight the infection. As the infection is suppressed and/or cleared, the effector cells either die or differentiate into memory CTL that can persist for long periods of time in the absence of antigenic stimulation. Models have been developed that explicitly take into account details of this differentiation pathway (Antia et al. 1998; Antia et al. 2005). Further, while the basic model assumes that CTL expansion is always proportional to the amount of antigen present, experiments have shown that in the initial expansion phase, an antigen-independent phase of "programmed proliferation" is observed, which has also been incorporated into models of CTL dynamics (Antia et al. 2003; Wodarz et al. 2000a; Wodarz and Thomsen 2005). To summarize, many developments of the basic model of CTL

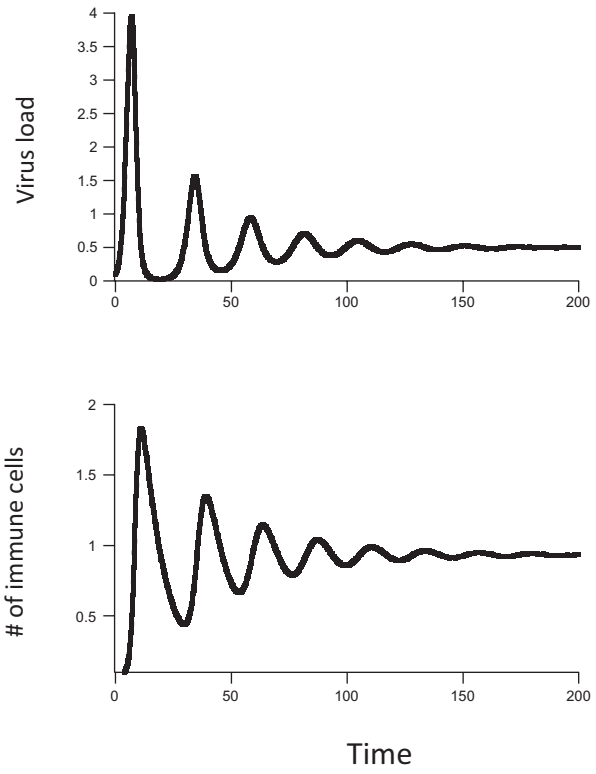

Fig. 2.1 Basic simulation of the dynamics between immune responses and an infection, based on model (1). Virus growth is followed by immune cell expansion, and immune cell-mediated activity reduces virus load. Subsequent damped oscillations bring the system towards an equilibrium. The level of virus load at this equilibrium shows how well the infection is controlled. If virus load lies below a threshold, this indicates virus clearance in practical terms. After expansion, the population of immune cells remains at an elevated memory level. The exact dynamics with which the equilibrium is approached can vary depending on the model and does not have much biological meaning. In this graph, there are prolonged oscillations before equilibrium is reached. Even if this is predicted by simple mathematical models, however, other factors not taken into account in the model, such as space, can reduce these oscillations and stabilize the dynamics

dynamics can be found in the literature, which however, are all rooted in and related to ecological population dynamics models.

These models have been applied to examine various biological questions. They have been fit to experimental data in order to measure important kinetic parameters of T cell responses (Perelson 2002; De Boer et al. 2001, 2003a, b, c; Bonhoeffer et al. 2000; Ribeiro et al. 2002a, b; Asquith et al. 2002; Ganusov et al. 2005). Various aspects of immune responses, such as the ability of a response to clear a pathogen (Nowak and Bangham 1996; Wodarz and Nowak 2000a; Chao et al. 2004), the potential for immune responses to cause pathology (Wodarz et al. 2002; Wodarz and Krakauer 2000), correlates of protection against re-infection (Antia et al. 1998, 2005; Borghans et al. 1999a; Wodarz et al. 2000b), and the dynamical interactions

between different branches of the immune system as well as between different immune cell clones (Nowak and May 2000; Borghans et al. 1999b; De Boer and Perelson 1994; Segel and Bar-Or 1999; Perelson et al. 1976; Bergmann et al. 1999, 2001; Yates et al. 2000; Scherer and Bonhoeffer 2005; Funk et al. 2005) have been investigated with mathematical models. Many other applications exist, which cannot all be reviewed here due to lack of space. Instead, the article will outline further aspects of CTL dynamics that are strongly related to ecological dynamics, in particular competition dynamics.

In the simple model described above, the immune cells were considered as a single population which responds to the pathogen and fights it. In reality, however, several species of immune cells can recognize the same pathogen and respond. They recognize different parts of the same pathogen. Not only do different branches of the immune system, such as antibodies and T cells, respond simultaneously. Even within one branch of the immune system, such as the killer T cells, different species or clones of cells recognize different parts of the same pathogen and respond. Thus, a diversity of immune responses develops. As explained above, the interactions between immune responses and pathogens can be considered a predator-prey system. In this analogy, there are different species of predators living off the same prey population. This means that the predators are in competition for their common food resource. The predator species which is most efficient at capturing the prey can reduce the food resource to levels which are too low for other predator species to survive. This can result in competitive exclusion where only one predator species remains. Similar considerations apply to the immune system (De Boer and Perelson 1994; Wodarz and Nowak 2000b; Nowak 1996; Nowak et al. 1995). If one immune cell clone is more efficient at responding to a virus, it can reduce virus load to levels which are too low for the other immune cell clones to become stimulated. This can result in the dominance of one immune cell clone and the exclusion of the others. This concept is called immunodominance. An immune response with a dominant immune cell clone is also called a narrow response. Another possible outcome of competition dynamics in ecology is coexistence. This can come about if there is a degree of niche separation between the species. Similarly, the coexistence of multiple immune cell clones can be observed. This is called a broad response, and different reasons can contribute to this outcome. One of the reasons might be the evolution of pathogens *in vivo* towards escape from immune responses (Nowak 1996; Nowak et al. 1995). Viruses can mutate the proteins which are recognized by the immune system. The mutant will initially grow unopposed. It is very likely, however, that another immune cell clone exists within the body which can recognize the mutant. This immune cell clone will become stimulated and expand. Now, two responses are present: the original response which controls the wild-type virus, and the new response which controls the mutant. Further mutants can arise, and further immune cell clones can become stimulated in order to fight the mutants. In this way, a broad immune response is found with many immune cell clones coexisting. Such non-equilibrium dynamics might be observed in some HIV infected patients (Nowak 1996; Nowak et al. 1995). Another factor which might determine whether a broad or a narrow immune response is observed could be the level of pathogen control in persistent infections. Mathematical studies (Wodarz and Nowak 2000b) suggest that efficient control of a persistent

infection correlates with a broad response, while less efficient control correlates with a narrow response. On a simplified level, the reason is that the competition between the immune cell clones is reduced in the presence of efficient control while this is not the case if the virus can replicate at higher levels. Efficient control can be brought about by a long life-span of CTL in the absence of antigen (low value of parameter *b*), which reduces the level of competition and can lead to a prolonged coexistence of several CTL clones, before extinction of the weaker clones is observed after a relatively long period of time. Yet, other studies have shown that the interplay between CTL responses and other branches of the immune system, such as helper cell responses, can lead to complex dynamics with multiple steady sates, in which the coexistence of different CTL clones can occur (Korthals Altes et al. 2003).

Just as different cell clones from a single branch of the immune system can compete against each other, so can different branches of the immune system (Arnaout and Nowak 2000; Wodarz 2003b). The two main branches which directly fight pathogens are antibody responses and killer T cell responses (CTL). Both populations expand when they are stimulated by a pathogen, and both responses work to remove the pathogen from the body. Antibodies can attack any pathogen which is present in the extracellular environment of the body. Killer T cells attack pathogens which are inside host cells (intracellular pathogens). These are mostly viruses. Viruses have both intra- and extra-cellular stages. Thus, the competition between antibody and killer T cell responses is mostly relevant in the context of viral infections. Mathematical modeling has investigated the basic competition dynamics between antibody and killer T cell responses (Arnaout and Nowak 2000; Wodarz 2003b). One of the outcomes is competitive exclusion. If antibodies are more efficient at fighting a virus, they can reduce virus load to levels which are too low to stimulate the killer T cells. Similarly, if killer T cells are more efficient at fighting the virus, they can reduce virus load to levels which are too low to stimulate the antibodies. In addition to these exclusion outcomes, there is also a coexistence outcome. Both antibodies and killer T cells persist and fight the infection. This is possible because they recognize different stages of the viral life cycle. The killer T cells are stimulated by intracellular virus, while antibody responses are stimulated by extracellular virus. For example, even if the killer T cells are very efficient at reducing the number of infected cells to low numbers, the population of free (extracellular) virus particles can still be sufficiently large to stimulate the antibody response. Such competition between killer T cells and antibodies might be observed in Hepatitis C virus (HCV) infection. A relatively small fraction of patients who become infected with HCV clear the virus from the blood and no further disease is observed. The rest of the patients develop persistent infection. This is initially asymptomatic, and the asymptomatic phase lasts for a long time. After about 10–20 years, liver pathology (hepatitis) is observed in a fraction of virus carriers. During the initial stages of infection (acute phase), both killer T cells and antibody responses expand. The role of killer T cells and antibodies for the resolution of HCV infection is debated in the literature (Farci et al. 2000; Klenerman et al. 2000; Cox et al. 2005a, b). Studies of the early phase of the infection showed that both humans or chimpanzees who cleared the virus from blood developed strong and sustained killer T cell responses (Thimme et al. 2001; Lechner et al. 2000a; Cooper et al. 1999; Lechner et al. 2000b, c;

Chang et al. 2001). In chimpanzee studies, killer cell-mediated clearance is associated with the absence of strong antibody responses (Cooper et al. 1999), and this supports the notion of competition between the two branches of the immune system. Humans and chimpanzees who developed persistent infection were characterized by an initial killer T cell response which was not sustained at high levels beyond the early phase of infection (Thimme et al. 2001; Lechner et al. 2000a; Cooper et al. 1999; Lechner et al. 2000b, c). Persistent infection has, however, been observed to be associated with vigorous antibody responses (Farci et al. 2000; Major et al. 1999), again pointing to the occurrence of competition. Thus, it was argued that a strong killer T cell response is crucial for the resolution of infection. It has been hypothesized that the progression of HCV infection from the asymptomatic phase to the pathogenic phase could be explained by the competition between antibody and killer T cell responses (Wodarz 2003b). As described above, persistent HCV infection is characterized by an ongoing antibody response, and by the absence of a significant killer T cell response. It is thought that HCV might be non-cytopathic. That is, the virus does not kill the cells it infects (the liver cells). Liver cell death would then require the activity of killer T cells. Since those are absent during the earlier stages of the infection, pathology is not observed. According to the ecological arguments presented here, the killer T cells are absent because they have been excluded by the antibody response. During the course of infection, HCV accumulates mutations and can evolve to escape the antibody responses. As the virus escapes antibody responses, the degree of immunological control is reduced and virus load rises. This provides increased levels of stimulation for the killer T cells. The more the virus evolves away from the antibodies (i.e. the higher the degree of viral diversity), the higher the level of stimulation of the killer T cell responses. Once the number of escape mutants has crossed some threshold, there is enough stimulation for the killer T cell population to expand. Now, the ongoing killer T cell response continuously removes infected liver cells, but fails to clear the infection. As a high percentage of liver cells tends to be infected by HCV, this can cause significant degrees of pathology (also called immunopathology (Thomsen et al. 2000; Chang et al. 1997; Zinkernagel and Hengartner 1994)). Therefore, virus evolution of antibody escape can shift the competition dynamics from a parameter region where the outcome is competitive exclusion of the killer T cells to another parameter region, where we observe coexistence between antibodies and killer T cells. This in turn may cause disease.

2.3 Immune Responses as Modulators of Virus Competition

The previous section mainly considered the dynamics of immune responses and ecological parallels in these dynamics. This section takes a more integrated approach and examines how immune responses can alter ecological interactions among pathogens and therefore play an important selective force in the *in vivo* evolution of pathogens. This will be done by considering a specific case study in the

context of HIV infection. If two virus strains share the same target cell population and are opposed by the same immune responses, standard models predict that the virus with the larger basic reproductive ratio excludes its competitor. Recent work, however, indicates, that viral competition dynamics can be significantly altered if models assume that multiple copies of the viral genome can infect the same cell (multiple infection or coinfection), and that immune responses can play an instrumental role in shaping the outcome of the dynamics.

Most work on virus/HIV dynamics was performed under the assumption that each cell is infected by a single copy of HIV. It is observed in vitro that upon infection, the virus induces down modulation of its receptor on the surface of the infected cell, rendering the cell resistant to further infection events. It has, however, become clear that multiple copies of HIV can infect the same cell, a process we call coinfection (Chen et al. 2005; Dang et al. 2004; Jung et al. 2002; Levy et al. 2004; Mattapallil et al. 2005; Gelderblom et al. 2008). While receptor down-modulation does occur (Lama 2003; Levesque et al. 2004), it only happens about 24 h post infection, leaving a time window that allows further copies of HIV to infect the cell (Lama 2003; Nethe et al. 2005). Further biological details about the process of coinfection are reviewed in (Wodarz and Levy 2011). Coinfection can have important consequences for the evolutionary dynamics of the virus *in vivo* (Gelderblom et al. 2008; Bonhoeffer et al. 2004; Fraser 2005; Vijay et al. 2008; Althaus and Bonhoeffer 2005; Kouyos et al. 2006, 2007; Iwabu et al. 2008; Dixit and Perelson 2004; Rouzine and Coffin 2005), for example through recombination between different genotypes that are packaged within the same virus particle.

However, coinfection can also have important consequences for the basic competition dynamics between different virus strains. This can be demonstrated in the context of the following model that is given by a set of ordinary differential equations (Wodarz and Levy 1999).

$$\begin{aligned}
\dot{x} &= \lambda - dx - \beta_1' x\left(y_1 + y_{12}\right) - \beta_2' x\left(y_2 + y_{12}\right) \\
\dot{y}_1 &= \beta_1' x\left(y_1 + y_{12}\right) - a_1 y_1 - \beta_2' y_1\left(y_2 + y_{12}\right) - p y_1 z \\
\dot{y}_2 &= \beta_2' x\left(y_2 + y_{12}\right) - a_2 y_2 - \beta_1' y_2\left(y_1 + y_{12}\right) - p y_2 z \\
\dot{y}_{12} &= \beta_1' y_2\left(y_1 + y_{12}\right) + \beta_2' y_1\left(y_2 + y_{12}\right) - a_2 y_{12} - p y_{12} z \\
\dot{z} &= cz\left(y_1 + y_2 + y_{12}\right) - bz
\end{aligned} \tag{2.2}$$

The variables y_1 and y_2 denote the populations of cells infected only by virus 1 and virus 2, respectively. The variable y_{12} denotes cells which are coinfected by both viruses. The variable z denotes a specific immune response which expands upon antigenic stimulation (such as B cell and T cells). We assume that virus 2 is more cytopathic than virus 1 (i.e. $a_2 > a_1$). Virus 1 replicates with a rate β'_1, and virus 2 replicates with a rate β'_2. It is assumed that the viral cytopathicity and replication rate are related according to $\beta'_1 = fa_1/(g + a_1)$ and $\beta'_2 = fa_2/(g + a_2)$. Virus 1 is produced from cells y_1 and y_{12}, and virus 2 is produced from cells y_2 and y_{12}. Cells infected with virus 1 die with a rate a_1, and cells infected with virus 2 die with a rate a_2. Cells infected with both viruses show dominance and die with a rate a_2 because

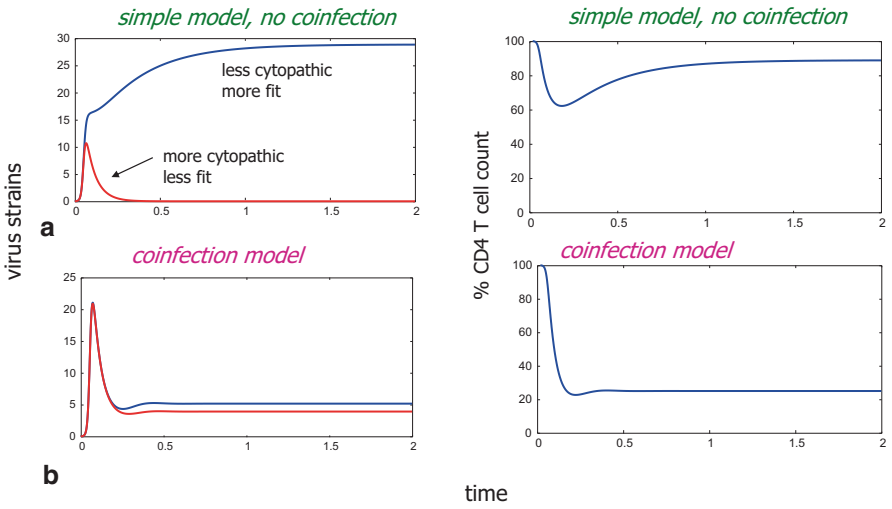

Fig. 2.2 Simulations showing the effect of HIV coinfection on the outcome of competition between a less cytopathic fitter strain and a more cytopathic strain that is less fit, based on model (2). **a** In the absence of coinfection, the fitter strain outcompetes the less fit strain. Because the fitter strain is weakly cytopathic, the CD4 T cell count is not predicted to dip significantly. **b** In the presence of coinfection, coexistence of the two virus strains is observed. Because the more cytopathic strain persists, the CD4 T cell count plunges to low levels

virus 2 is assumed to be more cytopathic than virus 1. Immune responses expand upon exposure to all types of infected cells. The simplest expression to describe this is given by $cz(y_1+y_2+y_{12})$, where c is the immune responsiveness (Nowak and May 2000). Further, immune cells die with a rate b. In the simplest form, the model assumes that immune responses kill infected cells with a rate p.

Assume that the basic reproductive ratio of each virus in isolation is greater than one. That is, in isolation, each virus would be able to sustain an infection. From now on, we assume that the cytopathicity of virus strain 1 is such that the basic reproductive ratio of the virus is at its maximum, i.e. this virus strain is characterized by maximum fitness ($a_1 = a_{fit}$). We further assume that virus strain 2 has a higher degree of cytopathicity ($a_2 > a_{fit}$) which enables it to induce more pronounced target cell depletion. As a result of these assumptions, the more pathogenic virus strain 2 is always less fit than the less pathogenic virus strain 1. We ask under which conditions the more cytopathic virus strain 2 can persist in the virus population. The less cytopathic virus strain 1 always persists. The more cytopathic virus strain 2 persists if $\frac{\beta'_2(x_1+y_1)}{a_2} > 1$, where $x_1 = a_1/\beta'_1$ and $y_1 = \lambda/a_1 - d/\beta'_1$. In other words, the less cytopathic strain can exclude the more cytopathic one, but not vice versa. If the above condition is fulfilled, a more cytopathic strain can coexist with a less cytopathic strain, even though it has a lower replicative fitness and would be driven extinct in the absence of coinfection (Fig. 2.2). The reason for the coexistence is as follows. Because the coinfected cells die with the rate determined by the more cytopathic virus,

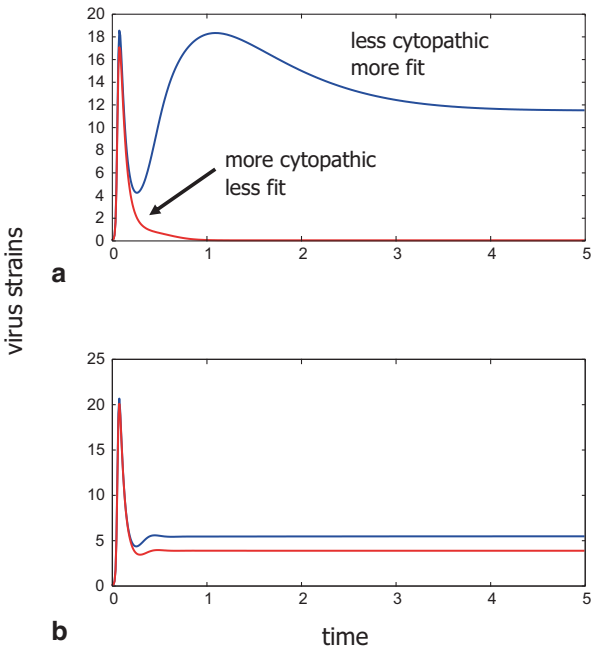

Fig. 2.3 HIV coinfection and the role of immune responses for strain competition, based on model (2). **a** When immune-mediated virus suppression is relatively strong, the fitter strain excludes its competitor because virus load is relatively low and coinfection does not play a driving role in the dynamics. **b** When immune-mediated virus suppression is weaker and virus load is higher, more coinfection occurs and coexistence of the two virus strains is observed

the advantage of being less cytopathic is largely abolished in the context of coinfection. Moreover, when coinfection is possible, cells infected with strain 1 remain targets for infection with strain 2 and vice versa, thus increasing the total number of susceptible cells. The persistence of competitively inferior pathogens has also been observed in epidemiological models that describe the spread of a pathogen through a population of hosts and that take into account the infection of a host by multiple pathogens (Levin 1983a, b; Levin and Pimentel 1981; Nowak and May 1994).

The result that a more cytopathic strain with reduced replicative fitness can coexist with a fitter virus strain has important implications. Even if the more cytopathic strain is subdominant, its presence can still result in a sharp decline of the overall number of target cells (CD4+ T cells). By how much the T cell count is expected to fall depends on the level of cytopathicity of the new strain.

If coinfection promotes the emergence of less fit and more cytopathic strains which induce disease, the question emerges why transmission to a new host always "resets the disease clock", i.e. results in the prevalence of less cytopathic HIV strains which do not cause disease, but keep the infection asymptomatic. The answer might be immune responses. For coinfection to be a significant force in the dynamics of HIV evolution and disease progression, virus load needs to be sufficiently high such that cells are frequently infected with more than one virus. If virus load is suppressed by immune responses, coinfection is a rare event and does not drive the dynamics. If the strength of immunity lies below a threshold, the dynamics are in principle the same as in the scenario where no immune responses are present. That is, coinfection allows the emergence of more cytopathic and less fit HIV strains which could lead to the depletion of the CD4 T cell population (Fig. 2.3). If

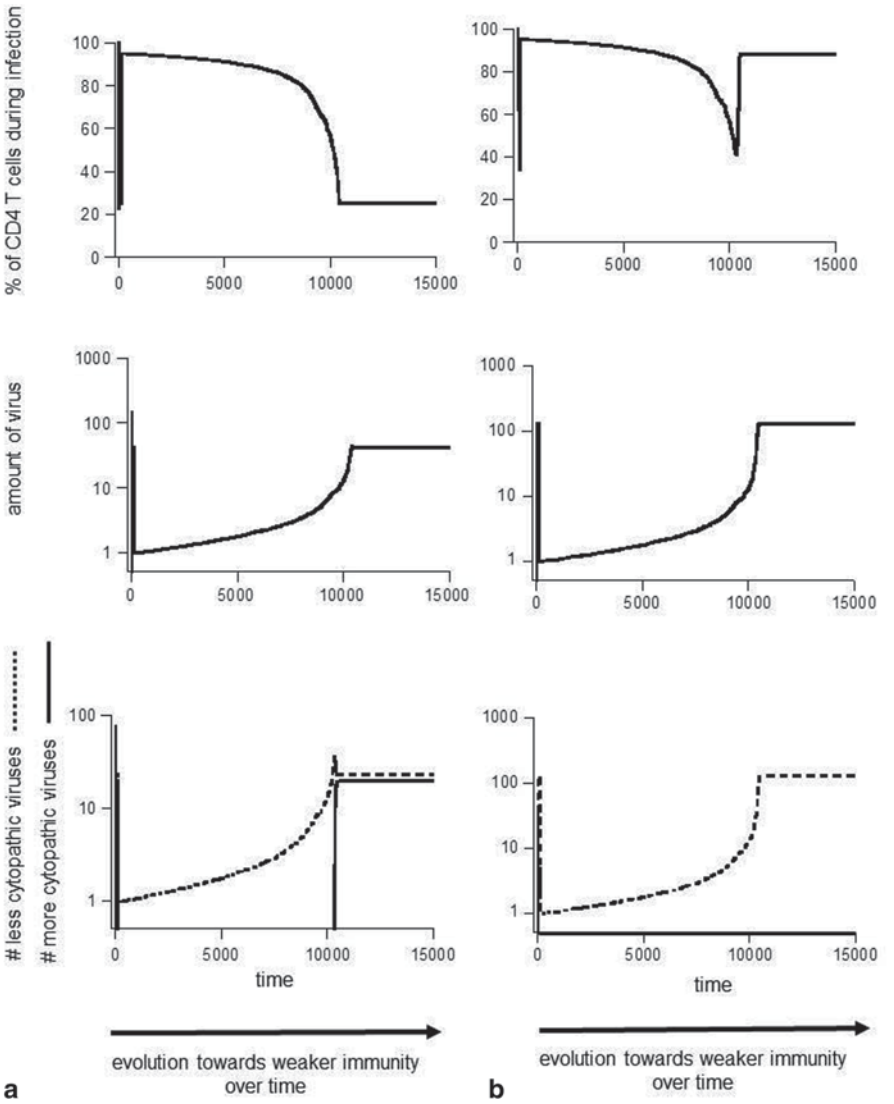

Fig. 2.4 Simulation of disease progression (**a**) assuming that coinfection can occur at sufficient levels, and (**b**) assuming that coinfection is blocked, based on model (2). These graphs plot the percentage of CD4 cells and virus load over time, assuming that the immune responsiveness, c, is reduced over time in a stochastic fashion. We start with the presence of a less cytopathic virus, and assume that at each time interval, there is a chance that a more cytopathic virus with a reduced basic reproductive ratio is created by mutation. **a** Coinfection dynamics: During the acute phase the virus replicates to high levels. Both virus strains can be present during the acute phase. The more cytopathic strain contributes to a temporary but significant drop in the CD4 cell count during this phase. As immune responses rise, virus load drops and the more cytopathic strain is out-competed by the less cytopathic strain with higher replicative fitness. This is because at low virus loads, the chance of coinfection occurring is relatively low. Consequently, the CD4 cell count returns to relatively high levels. Over time, the immune response weakens and virus load rises. Once virus

the strength of immunity is higher and lies above a threshold, however, the dynamics change fundamentally. Now, virus load is relatively low and coinfection is a rare event. Under this condition, the behavior of the model approaches the dynamics observed in the absence of coinfection. That is, more pathogenic and less fit viruses cannot emerge, and the less pathogenic, fitter variants prevail (Fig. 2.3). The virus population is also predicted to be more homogeneous since this condition favors competitive exclusion in general.

These results translate into the following overall picture of HIV disease progression, summarized with computer simulations in Fig. 2.4 and schematically in Fig. 2.5. During the acute phase where virus loads are high, the cytopathic strain persists and induces a temporary drop in the CD4 T cell count, which has been observed to occur especially in the gut-associated lymphoid tissue (Veazey et al. 1998). However, in the post-acute phase, relatively strong immunity is initially present, and this could account for the selection of less cytopathic strains which are characterized by higher fitness. Less fit and more cytopathic virus strains go extinct, and the patient remains asymptomatic. During progression, anti-viral immunity gradually loses its ability to control the virus because of a variety of reasons, such as the killing of HIV-specific helper cells (Kalams et al. 1999; Rosenberg et al. 1997), and viral escape from immune responses (Nowak et al. 1991; Price et al. 1997; Phillips et al. 1991; McMichael and Phillips 1997; Goulder et al. 1997; Kimata et al. 1999). Once the strength of immunity has fallen below a threshold level, virus load becomes sufficiently high such that coinfection is a frequent event and a driving force of the dynamics. In fact, it has been demonstrated experimentally that the amount of coinfection goes up according to the square of virus load (Levy et al. 2004). Now, more cytopathic and less fit virus strains can emerge and this can lead to the depletion of the CD4 T cell population and the development of AIDS (Figs. 2.4 and 2.5). If coinfection occurred at reduced levels, such cytopathic virus strains would not emerge, and this would result in the absence of disease, even at high virus loads (Fig. 2.4). In this case, virus load could still increase over time during the infection, for example as a result of immune escape, but the high virus load would not induce AIDS (Fig. 2.4).

If the virus gets transmitted from one host to another, immunity will once again be strong in the new host in the post-acute phase (Fig. 2.5). Consequently, coinfection is again a rare event. This once more results in the selection of virus strains which are not cytopathic enough to induce AIDS, and in the extinction of strains

load has risen beyond a critical threshold, a significant number of cells will be co-infected. This allows the more cytopathic strain with a reduced basic reproductive ratio to invade. This induces a relatively sharp and permanent drop in the CD4 cell count and could correspond to the development of AIDS. **b** Same simulation assuming that coinfection is blocked. The initial dynamics are identical. However, as immunity weakens and virus load rises to high levels, there is a fundamental difference: the more cytopathic strain with a reduced basic reproductive ratio cannot invade, and the CD4 T cell count is maintained at relatively high levels in the long term, despite the high viral loads. We observe a temporary drop in the CD4 cell count as the immune system weakens and virus load rises. This is because limited immune-mediated lysis in the presence of relatively high virus load can result in the killing of many CD4 cells, a process called CTL-induced pathology

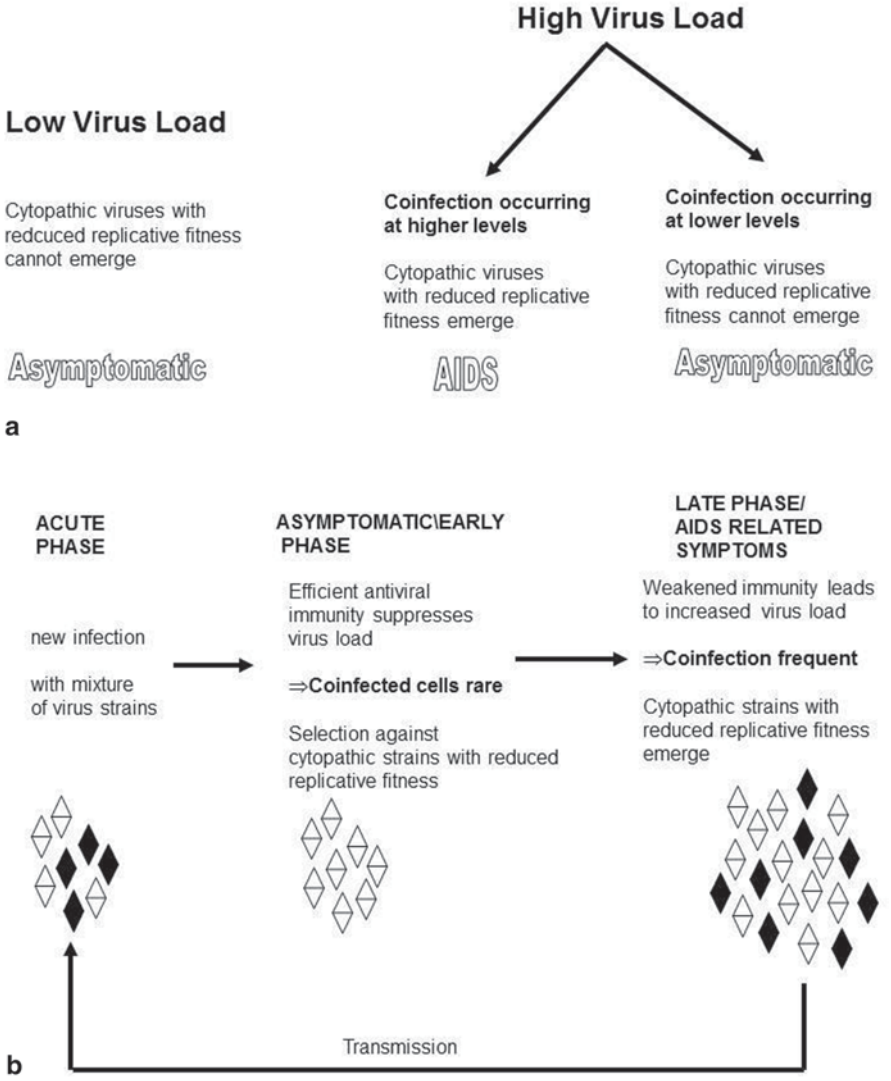

Fig. 2.5 Schematic diagram which summarizes the basic insights gained from the mathematical model. **a** At low virus loads, the more cytopathic strain with a reduced basic reproductive ratio cannot emerge because even if coinfection can take place, it is a rare event. This keeps the infection asymptomatic. At higher virus loads we have two possible outcomes. If coinfection can take place at sufficient levels, a more cytopathic strain with a reduced basic reproductive ratio can emerge, and this can result in depletion of the CD4 cell count and the development of AIDS. On the other hand, if coinfection occurs at reduced levels, more cytopathic strains with a reduced basic reproductive ratio cannot emerge, and so the infection remains asymptomatic despite high virus loads. **b** Schematic representation of the implications for HIV disease progression. A weakening of the immune responses can increase the frequency of coinfection and allow the rise of more cytopathic virus strains which have a reduced basic reproductive ratio and can induce AIDS. If a mix of virus strains is transmitted to a new host, the disease clock is reset, i.e. more cytopathic strains go extinct and the less cytopathic ones prevail, keeping the infection asymptomatic. This is because immune responses are once again strong in the new host which results in a drop of virus load, infrequent coinfection, and thus selection against more cytopathic and disease inducing strains which have a lower basic reproductive ratio

which are associated with the onset of disease. Hence, the clock of progression is reset and the disease is initially asymptomatic (Fig. 2.5).

Note that in this model, the escape from immune responses is not sufficient to induce AIDS. Escape from immune responses results in high virus loads. High virus loads, in turn, may or may not result in the development of AIDS. This depends on the level of coinfection that occurs, which determines whether disease inducing virus strains can emerge or not.

In the context of immune responses, it is important to clarify the statement that AIDS develops because the virus evolves towards reduced replicative fitness. For coinfection to be a significant driving force of the dynamics, the virus needs to first escape immune responses or weaken immune responses against itself. Obviously, this increases overall fitness of the viral population. However, once the immune response is weakened beyond a critical point and coinfection reaches a threshold level, then virus strains with reduced replicative fitness can emerge and induce an AIDS-defining level of CD4 depletion. By reduced replicative fitness we mean a reduction in the basic reproductive ratio of the virus, R_0, defined in the absence of coinfection, but only sustainable by the presence of coinfection made possible by weakened immunity. These concepts are explained in Fig. 2.5.

These results might also have implications for the reasons for the lack of AIDS in monkeys that are naturally infected with SIV. Naturally infected monkeys can show high viral loads, virus diversity, and lack of strong immune responses to SIV (Goldstein et al. 2000; Broussard et al. 2001; Silvestri et al. 2003)—factors associated with AIDS in HIV-infected patients. While the reasons for this remain unclear and subject to speculation, it is possible that in natural SIV infection, coinfection occurs at reduced levels. It is possible that either the virus or the host or both have evolved mechanisms which reduce the amount of coinfection. In this case, the virus would, according to our model, evolve to a phenotype which is not cytopathic enough to induce AIDS. HIV already has the ability to block coinfection, but only after the cell has been infected for a day or two. It is possible that this time window is significantly shorter in natural SIV infection. More experimental research is needed to investigate the molecular mechanisms which allow and prevent coinfection, and to compare natural SIV infection with pathogenic SIV/HIV infection in this respect.

2.4 Immune Responses and Immuno-Epidemiology

So far, the review has concentrated on the *in vivo* dynamics between immune responses and viruses. In this context, analysis typically focuses on the degree of immune-mediated virus control achieved, and stronger virus control or virus clearance is always thought of as the most desirable outcome. In most settings, this is also certainly the case. However, when the ecological interactions of the host species or the epidemiological dynamics of pathogens is taken into account, it is possible to find scenarios in which the situation is not so straightforward and in which it can be

beneficial for the host to develop suboptimal immunity to pathogens. Two examples will be discussed. In the first case, pathogen-mediated indirect interactions between two host species is discussed. In the second case, the correlation between the strain composition of pathogens and the degree of immune-mediated protection of hosts is discussed.

(a) Indirect Pathogen-Mediated Interactions Between Host Species This section explores the effect of immunity in two host species that compete and that are infected by their respective pathogens, to which they are adapted. In addition, it is assumed that the pathogen adapted to one host species can also infect the other host species to which it is not adapted, thus causing significantly higher pathogenic effects.

We consider a basic model which consists of four variables (Wodarz and Sasaki 2004). Uninfected and infected hosts of species 1 (x_1 and y_1), as well as uninfected and infected hosts of species 2 (x_2 and y_2). Uninfected hosts reproduce, die, and become infected by the pathogen. Infected hosts are assumed not to reproduce. They may die or recover from infection. When hosts recover from infection, they are assumed to become susceptible again (SIS model). The model is given by the following set of ordinary differential equations which describe the development of the populations over time.

$$\begin{aligned}
\dot{x}_1 &= \left[b_1 - d_1 - h_1\left(x_1 + x_2\right)\right]x_1 - \beta_1 x_1 y_1 - p_1 x_1 y_2 + \gamma_1 y_1 \\
\dot{y}_1 &= \beta_1 x_1 y_1 - \left(d_1 + \alpha_1 + \gamma_1\right)y_1 - q_1 y_1 y_2 \\
\dot{x}_2 &= \left[b_2 - d_2 - h_2\left(x_1 + x_2\right)\right]x_2 - \beta_2 x_2 y_2 - p_2 x_2 y_1 + \gamma_2 y_2 \\
\dot{y}_2 &= \beta_2 x_2 y_2 - \left(d_2 + \alpha_2 + \gamma_2\right)y_2 - q_2 y_2 y_1
\end{aligned} \tag{2.3}$$

where b_i, d_i and h_i are the birth rate, the natural mortality, and the coefficient of density dependent mortality of host species i; β_i, γ_i, and α_i are the transmission rate, the recovery rate, and the pathogenicity (additional mortality) of pathogen species i in its native host.

In addition to these basic infection dynamics, we also assume that the pathogen of one host species may infect the competitor (cross-species infection). That is, pathogen 1 (y_1) may infect host species 2 (x_2), and pathogen 2 (y_2) may infect host 1 (x_1). It is further assumed that this cross-species infection results in more pathogenicity than infection with the native pathogen (because there is lack of adaptation). In fact, we assume an extreme scenario for simplicity: Cross-species infection kills the competitor instantaneously, and the competitor cannot pass on the pathogen. In model terms, cross-species infection kills the uninfected and infected host populations at rates p_i and q_i, respectively.

In the following we describe the behavior of this model. Because we are interested in the effect of cross species infection on the competition dynamics, we assume that the two host species are competitively neutral in the absence of pathogens. That is, $b_1 = b_2$, $d_1 = d_2$, and $h_1 = h_2$. We further assume that the effect of cross-species infection is equivalent for the populations x and y; that is, $p_i = q_i$. In addition, the

killing rates are assumed to be identical for both host species. Because we discuss the system under these particular assumptions, we will only present the outcomes and equilibria which are relevant in this context. We define $K = (b - d)$ as the carrying capacity of either species of host. It is convenient to define the basic reproductive ratios, R_{01} and R_{02}, of pathogen species 1 and 2:

$$R_{01} = \beta_1 K / \left(d + \alpha_1 + \gamma_1\right)$$
$$R_{02} = \beta_2 K / \left(d + \alpha_2 + \gamma_2\right)$$

We observe the following outcomes.

(i) Disease-free equilibrium The pathogens are not maintained in the population. In this case, $y_1 = y_2 = 0$, and $x_1 + x_2 = K$, $K(1 - 1/R_{02}) < x_1 < K/R_{01}$. Because the host species are competitively neutral in the absence of pathogens, the sum of the two host species is kept at the carrying capacity K. The density of either host species must not exceed the threshold $x_1 < K/R_{01}$ and $x_2 < K/R_{02}$ in order to refuse the invasion of native pathogen species. The stable line segment defined above exists if $1/R_{01} + 1/R_{02} < 1$.

(ii) Species 1 wins and outcompetes species 2 The native pathogen is maintained in the population. That is, $x_2 = 0$, $y_2 = 0$, and $x_1 = K/R_{01}$, $y_1 = \frac{hK^2}{d + \alpha_1}\frac{1}{R_{01}}\left(1 - \frac{1}{R_{01}}\right)$. This equilibrium is locally stable if $1 < R_{01} < p_2 K/(d + \alpha_2)$.

(iii) Species 2 wins and outcompetes species 1 Again, the naïve pathogen is maintained in the population. That is, $x_1 = 0, y_1 = 0$, and $x_2 = K/R_{02}$, $y_2 = \frac{hK^2}{d + \alpha_2}\frac{1}{R_{02}}\left(1 - \frac{1}{R_{02}}\right)$

The equilibrium is locally stable if $1 < R_{02} < p_1 K/(d + \alpha_1)$.

(iv) Coexistence equilibrium Both species coexist and the respective pathogens are maintained ($x_1 > 0$, $y_1 > 0$, $x_2 > 0$, $y_2 > 0$). Equilibrium expressions are complex and not written out here. This outcome is observed if $R_{01} > p_2 K/(d + \alpha_2)$ and $R_{02} > p_1 K/(d + \alpha_1)$.

We are interested in how the rate of recovery from infection, γ_i, influences the competitive ability of the hosts. The stability properties of the equilibria in dependence of the recovery rate for the two species (γ_1 and γ_2) are shown schematically in Fig. 2.6. The thresholds which separate different outcomes are derived from the stability conditions given above. In the following we will ignore the disease free outcome, since we are interested in the effect of the pathogens. Coexistence of the two host species is only possible in a limited parameter region when the recovery rates of both species are low. Otherwise, we observe the following pattern. If the recovery rate of species 1 lies below a threshold relative to that of species 2, species 2 wins. The cost of carrying the pathogen in the population outweighs the benefit. If the recovery rate of species 1 is high and lies above a threshold relative to species 2, species 2 wins again. This is because a high recovery rate keeps the pathogen at

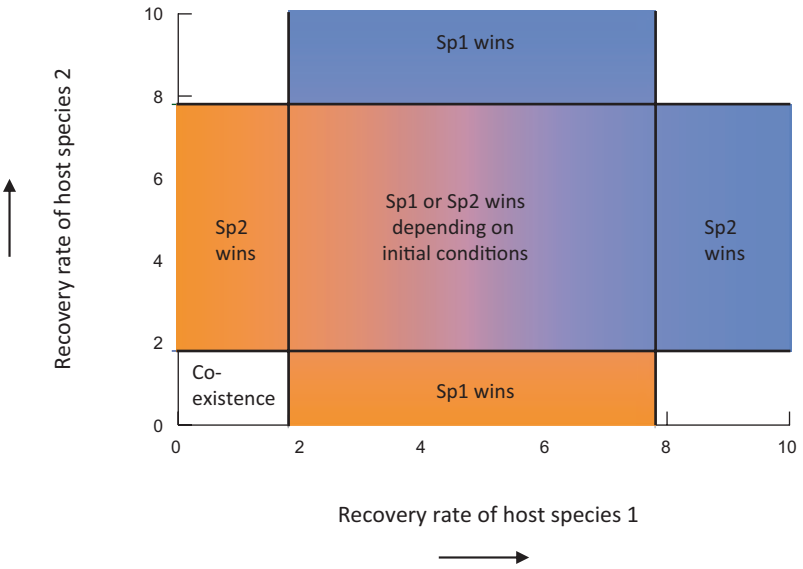

Fig. 2.6 The effect of the recovery rate from infection on the competition dynamics of two host species (Sp1 and Sp2) which share two species of parasites, based on model (3). The thresholds separating the different parameter regions have been calculated from expressions derived from the mathematical model but are not shown here. They depend on parameters which determine the basic reproductive ratio of the pathogens, and on the degree of host mortality upon cross-species infection. The parameter regions which are left blank represent outcomes which are not relevant for the present context and which are not discussed

low levels, allowing for only little transmission to the competitor. If the recovery rate of species 1 is intermediate (between the thresholds cited above), it can win and outcompete species 2. The pathogen is prevalent enough to be used as a biological weapon, while it is not too costly for the native host. If the recovery rates of both species are intermediate (parameter space including the orange to blue gradient in Fig. 2.6), then two outcomes are stable: either species 1 wins, or species 2 wins, depending on the initial conditions. The higher the initial abundance of one species relative to the other, the more likely this species is to win and persist. In the region of bistability, the exact initial abundance of the host species required to win the competition depends in the recovery rate from infection. The higher the recovery rate, the higher the initial abundance required to outcompete the other host species. This is because a higher recovery rate compromises the use of the parasite as a weapon in host competition.

This analysis demonstrates that in a more complex ecological setting, the role of immunity and pathogen control becomes less straightforward. On the one hand, efficient immune control is advantageous because it reduces the mortality of the host. On the other hand, it can also be disadvantages because it reduces the ability of the host population to transmit the pathogen to a competing host species in which it causes higher degrees of virulence. However, these arguments are based

on the advantage of the group and not the individual. If evolutionary dynamics are taken into account (Wodarz and Sasaki 2004), a mutant within a given species that is characterized by increased pathogen control will always invade, and the species will evolve towards higher degrees of immune protection. As shown by the model, this can lead to the evolution towards extinction of the species if it interacts with another species that carries a pathogen which can be transmitted across the species barrier and which cause high degrees of virulence.

(b) Immuno-Epidemiology This section is concerned with the evolution of immunological memory, defined as the duration for which hosts are protected from re-infection. Typically, after a host is infected with a given pathogen and has cleared the pathogen, specific immune cells remain at elevated levels and are very long lived in the absence of the pathogen (Ahmed and Gray 1996; Zinkernagel 2000). When the same pathogen infects the host a second time, this immune memory protects against re-infection and the development of symptoms. Traditionally, it is thought the a longer duration of memory is advantageous for the host. Here, this is discussed within a theoretical framework in two settings: where a host can be infected by a single pathogen strain, and where two pathogen strains with different levels of virulence co-circulate in the host population. The theoretical takes into account susceptible, infected, and recovered/immune hosts. In the presence of only one pathogen strain, the model is given by the following set of ordinary differential equations (Wodarz 2003a), where S denotes the number of susceptible hosts, I the number of infected hosts, R the number of recovered hosts that are protected against re-infection, and P the pathogen population.

$$\begin{aligned}
\dot{S} &= \frac{r(S+I+R)}{\varepsilon(S+I+R)+1} - dS - \beta SP + gR \\
\dot{I} &= \beta SP - aI - \alpha I \\
\dot{R} &= \alpha I - dR - gR \\
\dot{P} &= kI - uP
\end{aligned} \tag{2.4}$$

Uninfected and susceptible hosts are assumed to reproduce at a rate r and die at a rate d. They become infected by the pathogen at a rate β. Infected hosts are characterized by an elevated death rate, a, reflecting pathogen-induced mortality. In addition, they are assumed to recover from infection at a rate α. Recovered hosts die at the same rate as uninfected individuals, d, and they cannot be re-infected by the pathogen. This protection is not infinite, but is lost at a rate g. The model assumes that all host populations reproduce. The pathogen is, however, not transmitted vertically to the offspring. Moreover, it is assumed that offspring from recovered and immune hosts are once again susceptible to infection. (while antibody memory can be transferred from mother to child, this protection only lasts for a few months; T cell memory is not transferred from mother to offspring). Finally, pathogens can be released from the hosts into the environment at a rate k, and may decay in the environment at a rate u.

Since the model, immune hosts revert to being susceptible at a rate g; the duration of memory is thus given by $G = 1/g$. In the context of this model, mathematical analysis clearly shows that a longer duration of memory is always advantageous for the host. In the model, the duration of memory is predicted to evolve to infinity ($G = \infty$), but in reality it would of course be finite, determined by reproductive tradeoffs not included in the model. We refer to this as the maximum memory outcome.

Now, consider a more complicated scenario which describes the interactions between two pathogen species or strains (Pevear et al. 1999; Groarke and Pevear 1999) which compete for one host population. An important assumption is that the two pathogen species or strains are not immunologically cross-reactive. Therefore, hosts recovered from one pathogen are still susceptible to infection by the other. The two populations of pathogens are denoted by P_1 and P_2. The population of hosts infected with pathogen 1 are denoted by I_1, and hosts recovered and immune to pathogen 1 are denoted by R_1. Similarly, hosts infected by pathogen 2 are denoted by I_2, and hosts recovered and immune to pathogen 2 are denoted by R_2. Hosts immune to pathogen 1 are still susceptible to pathogen 2, and hosts immune to pathogen 2 are susceptible to pathogen 1. Thus, we have the following additional populations: Hosts recovered from pathogen 1 and infected with pathogen 2, I_{12}; hosts recovered from pathogen 2 and infected with pathogen 1, I_{21}. Hosts recovered and immune to both infections are denoted by R_{12}. For simplicity, it is assumed that hosts do not experience simultaneous multiple infections. The equations for the model are given as follows (Wodarz 2003a):

$$\begin{aligned}
\dot{S} &= \frac{rH}{\varepsilon H + 1} - dS - \beta_1 S P_1 - \beta_2 S P_2 + g\left(R_1 + R_2 + R_{12}\right) \\
\dot{I}_1 &= \beta_1 S P_1 - a_1 I_1 - \alpha_1 I_1 \\
\dot{R}_1 &= \alpha_1 I_1 - dR_1 - gR_1 - \beta_2 R_1 P_2 \\
\dot{I}_2 &= \beta_2 S P_2 - a_2 I_2 - \alpha_2 I_2 \\
\dot{R}_2 &= \alpha_2 I_2 - dR_2 - gR_2 - \beta_1 R_2 P_1 \\
\dot{I}_{12} &= \beta_2 R_1 P_2 - a_2 I_{12} - \alpha_2 I_{12} \\
\dot{I}_{21} &= \beta_1 R_2 P_1 - a_1 I_{21} - \alpha_1 I_{21} \\
\dot{R}_{12} &= \alpha_2 I_{12} + \alpha_1 I_{21} - dR_{12} - gR_{12} \\
\dot{P}_2 &= k_2\left(I_1 + I_{21}\right) - uP_1 \\
\dot{P}_2 &= k_2\left(I_2 + I_{12}\right) - uP_2
\end{aligned} \tag{2.5}$$

where the sum of the host population is given by $H = S + I_1 + R_1 + I_2 + R_2 + I_{12} + I_{21} + R_{12}$. The model gives rise to the interesting finding that the duration of memory (G) can regulate the outcome of competition between the two pathogens (Fig. 2.7). If the duration of memory is short and the population of immune hosts becomes susceptible again at a fast rate (low value of G), competition between pathogens is strong and the superior pathogen wins and excludes the inferior one. On the other hand, if the duration of protection is longer (higher value of G), competition between the two pathogens is weaker and coexistence of the pathogens can be observed

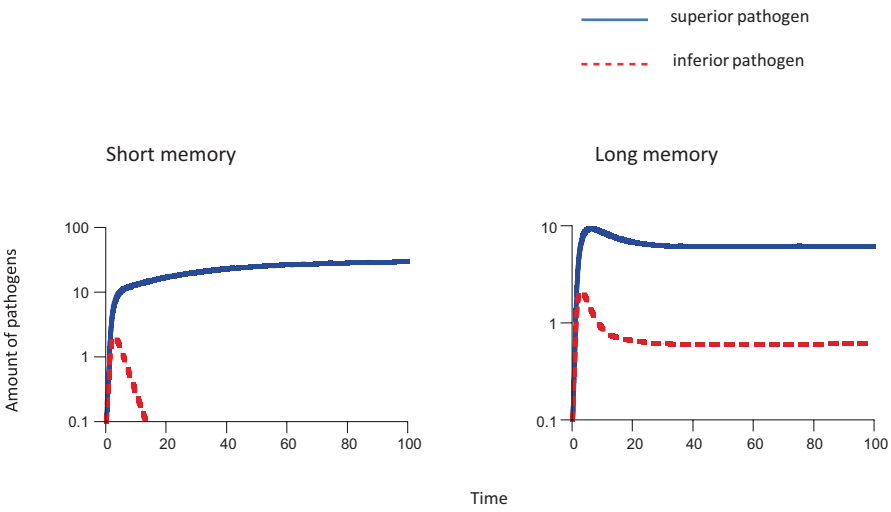

Fig. 2.7 Duration of memory and the competition between an inferior and a superior pathogen, based on model (5). If memory is short, the superior pathogen out-competes and excludes the inferior one. If the duration of memory lies above a threshold, the inferior pathogen can co-exist with the superior one

(Fig. 2.7). The longer the duration of protection, the higher the relative abundance of the competitively inferior pathogen. In other words, long lasting immunological memory allows a competitively inferior pathogen to persist and to be maintained in the host population. The reason is as follows. A long duration of memory results in the presence of hosts which are only susceptible to one, but not the other pathogen species. Therefore, the degree of interspecific competition is reduced relative to intraspecific competition. This results in coexistence. If, on the other hand, the duration of memory is relatively short, then the majority of hosts will be susceptible to both pathogen species. In this case, the degree of interspecific competition is much higher and competitive exclusion is observed.

What are the implications of these findings for the evolution of immunological memory? The answer depends on the assumptions of the model. If the inferior pathogen species is less virulent, persistence of this pathogen due to a longer duration of memory can only be advantageous for the host. The situation is, however, more complicated if the inferior pathogen is more virulent. In this case, the persistence of the inferior pathogen due to a longer duration of memory can be costly for the host population because the level of virulence is higher, and the following concentrates on the scenario described below.

In this parameter region where the inferior pathogen is more virulent, the following arguments apply. While a long duration of memory is advantageous because it protects the host from re-infection, a short duration of memory can also be advantageous because it allows less virulent pathogens to exclude more virulent ones. We observe two outcomes towards which the system may evolve (Fig. 2.8). One of the outcomes is maximum memory ($G = \infty$). The other outcome is a suboptimal and

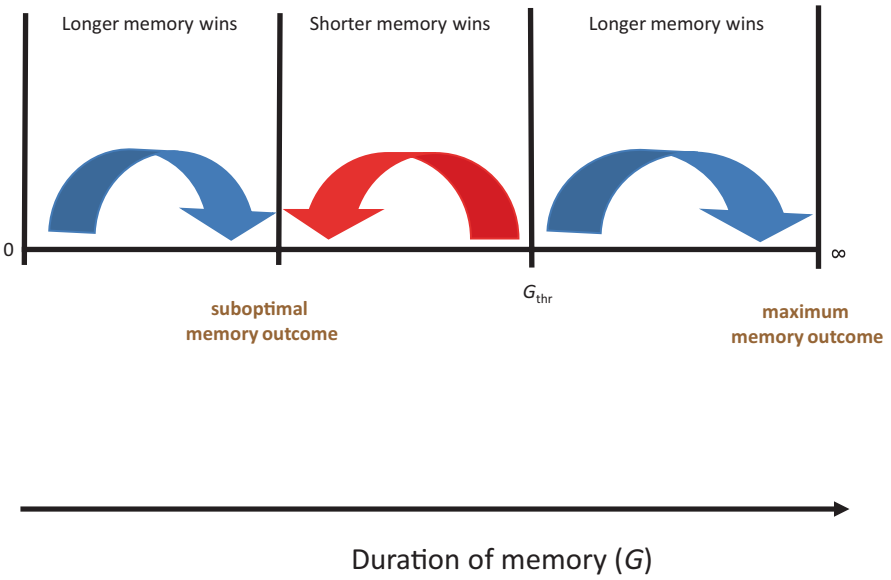

Fig. 2.8 Schematic representation depicting the evolution of immunological memory assuming the presence of two populations of pathogens which differ in their fitness, based on model (5). The pathogens differ in their virulence (rate of host killing), and we assume that we are in a parameter region where increased virulence correlates with reduced fitness. The system can evolve towards to different outcomes. The maximum memory outcome and the shorter or "suboptimal" memory outcome. Which outcome is achieved by evolution depends on the initial conditions; that is the initial duration of memory. If the simulation is started with an initial duration of memory which lies above G_{thr}, having a longer duration of memory is advantageous. Thus, evolution takes the system to the maximum memory outcome. If the simulation is started with an initial duration of memory which lies below G_{thr}, the system evolves towards the suboptimal memory outcome. The system can converge to the sub-optimal memory outcome for the following reason. If the duration of memory lies below G_{thr} but above the suboptimal memory outcome, a shorter duration of memory is advantageous and wins. If the duration of memory lies below the suboptimal memory outcome, longer memory is advantageous and wins

shorter duration of memory (smaller value of G). To which state the system evolves depends on the starting condition, G_0 (Fig. 2.8). If we start with a duration of memory which lies above a threshold ($G_0 > G_{thr}$), the system evolves towards maximal memory. If we start with a duration of memory which lies below the threshold ($G_0 < G_{thr}$), the system evolves to the state describing suboptimal memory. The initial duration of memory which separates the two outcomes (value of G_{thr}) depends on the rate of host killing by the more virulent pathogen. At one extreme, the rate of host killing by the more virulent pathogen is similar to that of the less virulent pathogen. In this case, the threshold duration (G_{thr}) separating the two outcomes is short. Therefore, the system is likely to evolve to maximum duration of memory. The reason is that the difference in virulence between the two pathogens is low. Thus, it does not pay to reduce memory in order to exclude the more virulent pathogen. At the opposite extreme, the rate of host killing by the more virulent pathogen is much higher than that of the less virulent one. Now, the threshold duration (G_{thr}) which separates

the two outcomes is very high. In this case, the system is likely to evolve towards the suboptimal memory outcome. The reason is that the more virulent pathogen is characterized by a much higher rate of host killing compared to the less virulent one. It therefore confers a significant cost to the host population, and exclusion of the more virulent pathogen by means of memory reduction confers a significant advantage. Note, however, that the duration of protection at the suboptimal memory outcome becomes longer as the rate of host killing by the more virulent pathogen is increased. The reason is that a higher rate of host killing reduces the relative fitness of the more virulent pathogen, and thus less memory reduction is required to exclude it.

Note, however, that the two outcomes to which the system can evolve may not be stable states. Assume that evolution takes the system to the suboptimal memory outcome. This can result in the exclusion of a more virulent pathogen and reduction in pathogen diversity. As pathogen diversity is reduced, it will become advantageous again to evolve a longer duration of memory because this leads to lasting protection. As memory becomes longer, however, inferior pathogens may invade again. As a consequence more virulent pathogens can persist and pathogen diversity increases. In this scenario, it will once again pay to evolve towards a shorter duration of memory. Thus, we may expect the duration of memory to cycle over time.

Similar to the previous case study, this example again shows that in an ecological context, the effect of immune protection on the mortality and fitness of hosts is not straightforward, but can be rather complex, depending on the exact circumstances under consideration.

2.5 Conclusion

This review has examined the relationship between immunology, ecology, and evolution on two distinct levels. On the one hand, the interactions between components of the immune system and infectious agents can be viewed as an *in vivo* ecosystem, the dynamics of which can be described by population dynamic models that have their roots in population ecology. The application of different ecological concepts was discussed, and a specific case-study was considered as an example to show how immune responses can modulate the complex *in vivo* evolutionary dynamics of pathogens. On the other hand, the review examined how the role of immunity and evolutionary considerations can be influenced when considered in a broader ecological setting where different host species interact in the face of one or more circulating pathogen(s). It was shown that the survival of individuals in the face of pathogens does not have to be enhanced by the strongest level of pathogen control, but that the immune system can in fact be used to modulate the ecological interactions among different pathogens, and that a weaker immune system can sometimes lead to improved survival. Overall, research approaches that span not only disciplines but also different levels of biological interactions are very valuable for furthering our understanding of the many questions in this field of research.

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Chapter 3
Reproductive and Immune System Interactions in the Context of Life History and Sexual Selection Theory

Kenneth M. Fedorka

Abstract Host variation in parasite load abounds, both within and across natural populations. The forces that shape and maintain this variation, however, are much less obvious. Over the past two decades, the emerging field of ecoimmunology has begun to address the underlying sources of this variation and its evolutionary consequences. Clearly, spatial and temporal heterogeneity in the environment contribute to variation in host parasite load by varying parasite distribution and abundance. However, host variation in the ability to acquire resources, and differences in how these resources are allocated, also play an integral role in parasite susceptibility. That is to say, not all individuals within a population are capable of managing the cost of immune defense, or may employ different cost-managing strategies. This realization has spurred a surge in studies focused on how immune pathways compete with other costly physiological pathways, such as those associated with reproduction. Interest in this relationship has no doubt been driven by the reproductive system's high energetic cost and its direct association with host fitness. In this chapter, I examine the interactions between reproduction and immunity to highlight the simultaneous role both play in the evolution of immunological and reproductive adaptations. I begin by placing this interaction in the context of life history theory and discuss how competition for limited resources may constrain the evolution and expression of both systems. I then discuss the role that parasites play in directly shaping reproductive adaptations through sexual selection. In this discussion, I attempt to shed light on the lingering controversy that has overshadowed genetic benefit models and provide concrete predictions for future directions.

Keywords Life history theory · Evolutionary trade-offs · Physiological trade-offs · Parasite mediated sexual selection · Hamilton-Zuk hypothesis

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3_3,

3.1 Immunity in the Context of Life History Theory

The goal of all organisms is to reproduce. How they reach this goal, however, is variable. For instance, organisms may differ in the age or size at first reproduction; in how many times reproduction is attempted; or in how much energy is allocated to a given reproductive event. Thus, there are many paths to Nirvana. Life history theory provides a theoretical framework to explain the evolution of these many paths. At its core, life history theory operates under the basic tenet that organisms represent an optimal compromise between suboptimal traits (Roff 2002; Stearns 1992; Roff 1992). This compromise arises from the fact that fitness associated traits are costly and that the resources needed to fully express these traits are limited. Individuals are therefore forced to allocate their constrained resources among competing characters, which will result in a negative statistical association between traits known as a "life history trade-off". Trait optimality is rarely achieved considering that organisms exist in a variable world where resources are finite and optimal trait values remain dynamic.

One of the most well characterized life history trade-offs is that between reproductive effort and survival, which has been documented across plants, birds, reptiles, mammals and insects (Roff 1992; Stearns 1992). For instance, individuals who are experimentally prohibited from mating tend to live longer than those allowed to mate (Fedorka et al. 2004; Gems and Riddle 1996; Partridge and Farquhar 1981). Brood manipulations in passerine birds have also demonstrated that increased parental effort often leads to a truncated lifespan (Askenmo 1979; Boyce and Perrins 1987; Gustafsson et al. 1994; Norris et al. 1994). Although these patterns have been reported in the literature for some time, the proximate mechanisms underlying them have largely eluded us. A promising possibility is that mating-induced immune suppression is a key mediator in the antagonistic relationship between reproduction and survival (Sheldon and Verhulst 1996; Harshman and Zera 2007; Fedorka et al. 2004). To explore this proposition, however, we must first demonstrate that immunity is costly.

3.1.1 The Cost of Immunity

The fact that many immunological pathways are not constitutively expressed, but must be induced upon infection, implies that immunity has costs (Lazzaro and Little 2009; Frank 1993). These costs may be either evolutionary or physiological in origin. This distinction is important because it may have different implications for the evolution of, or the expression of, immunological and reproductive characters, respectively (Schmid-Hempel 2003). Evolutionary costs of immunity arise from negative genetic correlations between immunological traits and other fitness-related characters (Roff 1997). The consequence of this antagonistic genetic architecture is that the evolution of immunity is constrained by selection elsewhere in the organism. This is clearly evident in the fruit fly, *Drosophila melanogaster*, where selection for improved resistance to parasitoid attack resulted in flies that were

Table 3.1 Immune system evolution is constrained by an evolutionary trade-off with the reproductive system

Species	Manipulation	Reproductive/immunological effect	Reference
Reproductive selection			
Scatophaga stercoraria (dung fly)	Increased levels of polyandry; increased reproductive organs	Reduced phenoloxidase activity (♀, ♂)	Hosken (2001)
Aedes aegyptii (mosquito)	Earlier age at first reproduction	Reduced encapsulation response (♀)	Koella and Boëte (2002)
Immune selection			
Biomphalaria glabrata (snail)	Increased resistance to trematode *Schistosoma mansoni*	Reduced fecundity (♀)	Webster and Woolhouse (1999)
Plodia interpunctella (indian meal moth)	Increased resistance to granulosis virus infection	Reduced egg viability (♀)	Boots and Begon (1993)
Armigeres subalbatus (mosquito)	Increased resistance to nemotode *Brugia malayi*	Delayed oviposition, ovary protein content (♀)	Ferdig et al. (1993)
Drosophila melanogaster (fruit fly)	Increased resistance to parasitoid *Asobara tabida*	Increased mating success (♂)[a]	Rolff and Kraaijeveld (2003)
Aedes aegyptii (mosquito)	Increased resistance to protist *plasmodium gallinaceum*	Reduced fecundity (♀)	Yan et al. (1997)
Gallus domesticus (domestic chicken)	Increased antibody response to sheep erythrocytes	Reduced comb size, a sexually selected ornament (♂)	Verhulst et al. (1999)
Pedigree Analysis			
Teleogryllus oceanicus (field cricket)	Quantitative genetic half-sibling analysis	Negative genetic correlation between sperm viability and lysozyme activity (♂)	Simmons and Roberts (2005)
Drosophila melanogaster (fruit fly)	Quantitative genetic hemiclone analysis	Negative genetic correlation between bacterial resistance and fecundity (♀)	McKean et al. (2008)

[a] contrary to trade-off expectation

inferior larval competitors (Fellowes et al. 1998; Kraaijeveld and Godfray 1997). Although strong evidence for evolutionary costs to immunity exists (Table 3.1; Sutter et al. 1968; Bayyari et al. 1997; Bisset et al. 2001), detecting these costs may prove difficult if the negative genetic correlation appears only during certain life stages. For instance, mutations that enhance early age reproduction may come at the cost of immunosenescence later in life (a.k.a. antagonistic pleiotropy; Franceschi et al. 2000; Kirkwood and Rose 1991).

Alternatively, physiological costs may stem from both the maintenance of the immune system in the absence of infection (i.e. investing in the infrastructure to sense and respond to potential invaders) and deployment of the immune effectors once an invader is detected. Part of these costs may be derived from collateral damage of host tissue caused by immune effectors during deployment (a phenomenon known as immunopathology; Graham et al. 2005). However, they may also stem from the energetic expense of maintaining and deploying the immune system; energy that could be otherwise used for demands such as gametogenesis or mate searching. These types of costs are often represented in life history theory using the Y-Model of resource allocation (Zera and Harshman 2001), where each allocation decision results in the bifurcation of energy between one physiological path or another. It is this bifurcation that induces the physiological life history trade-off. If additive genetic variation exits for the physiological bifurcation process, then selection can shape the relationship and an evolutionary trade-off will evolve. I discuss the importance of these trade-offs in the following section.

3.1.2 Reproduction and Immunity Trade-Offs

3.1.2.1 Evolutionary Trade-Offs

As stated earlier, the trade-off between immune and reproductive systems is of great interest to biologists since they represent the two most significant components of individual fitness, survival and reproductive success. Evolutionary costs are of particular interest because negative pleiotropic relationships can act to constrain trait response to selection, assuming the magnitude of selection is similar for each trait (Lynch and Walsh 1998; Roff 1997). Thus, a negative genetic correlation between immune and reproductive pathways can have a profound influence on the evolution of disease resistance. To determine the extent of this constraint, biologists generally employ two basic methods grounded in quantitative genetic theory. The first employs the power of pedigree analysis to uncover the genetic variance—covariance matrix of reproductive and immune related traits (Roff 1997). Kurt McKean and colleagues utilized a variant of this approach (hemiclone analysis) to uncover a negative genetic correlation between bacterial resistance and fecundity in female *Drosophila melanogaster* (McKean et al. 2008). Although this is an effective method, it is relatively underutilized. Instead, researchers tend to employ experiments where either a reproductive or immunological character is selected for increased or decreased expression over successive generations. For instance, continued selection for increased resistance to the protist, *Plasmodium gallinaceum*, resulted in a significantly reduced female fecundity in the mosquito *Aedes aegyptii* (Yan et al. 1997).

Although these studies provide support for potential antagonistic coevolution between reproductive and immunological traits (Table 3.1), important caveats exist that must be addressed. First, these methods are labor intensive and/or time

consuming, resulting in a paucity of studies that examine the evolutionary cost of immunity under natural conditions. Instead, the preponderance of work has focused on organisms either easily manipulated in the laboratory, or with relative quick generation times. Second, selection experiments tend to employ relative large selection coefficients within the backdrop of a benign laboratory environment in order to obtain the desired phenotypic effect. This design will favorably propagate mutations of large effect somewhat independent of their antagonistic pleiotropic effects (Lazzaro and Little 2009). Therefore, selection experiments may overestimate the potential for antagonistic coevolution between reproductive and immune systems in the wild, considering that selection is likely to be less severe and strong negative pleiotropic effects will be less tolerated.

One solution to these caveats is to estimate evolutionary constraints directly in the field using non-manipulative techniques. This could be accomplished by employing a pedigree-free experimental design, where pair-wise relatedness among individuals is estimated using molecular markers (Ritland 1996; Lynch and Ritland 1999). These same individuals can then be assessed for a variety of reproductive and immune related traits, allowing the estimation of genetic correlations between any two characters of interest. Although pedigree-free approaches have limitations (Frentiu et al. 2008), they are a relatively simple and effective method for estimating quantitative genetic parameters. Adoption of this approach could provide a wealth of information regarding the evolutionary constraints placed on the immune systems of wild organisms. Unfortunately, the approach has not been wildly employed.

3.1.2.2 Physiological Trade-Offs

When assessing the potential for a physiological trade-off, two basic approaches are commonly utilized. The first approach is strictly observational, and assesses the potential for a trade-off by simultaneously measuring the reproductive output and immunological status of an individual (e.g. Festa-Bianchet 1989; Skarstein et al. 2001). Although this type of study can provide useful information, it makes drawing definitive conclusions difficult due to its correlative nature. A second and more informative approach is to experimentally manipulate one of the two characters of interest. This has most commonly been achieved by manipulating reproductive effort; specifically, by altering the number of mating opportunities in insects or brood size in birds (Table 3.2). A recent meta-analysis examined the effect of brood and clutch size manipulation on either the responsiveness to a novel immune elicitor (e.g. injection with phytohaemagglutinin) or the level of circulating blood parasites (Knowles et al. 2009). The resulting meta-regression, which included 26 independent bird studies, revealed that increased reproductive effort was negatively associated with the degree of immunological response to novel antigens. Moreover, they identified a positive association between reproductive effort and the degree of parasites circulating in the blood, indicating that increased investment in reproduction generally leads to reduced immune function.

Table 3.2 Immune system expression is constrained by a physiological trade-off with the reproductive system

Species	Manipulation	Reproductive/immunological Effect	Reference
Reproductive manipulation			
Allonemobius socius (ground cricket)	Increased mating opportunity	Reduced encapsulation abiltiy and lytic activity (♀, ♂)	Fedorka et al. (2004)
Drosophila melanogaster (fruit fly)	Increased mating opportunity	Reduced bacterial clearance ability (♂)	McKean and Nunney (2001)
Matrona basilaris (damselfly)	Increased mating opportunity	Reduced encapsulation abiltiy (♀, ♂)	Siva-Jothy et al. (1998)
Gryllus texensis (field cricket)	Increased mating opportunity	Increased resistance to Seratia marcescens (♀)[a]	Shoemaker et al. (2006)
Hirundo rustica (barn swallow)	Tail elongation (sexually selected trait)	Reduced gamma-globiulin concentration (♂)	Saino and Møller (1996)
Ficedula albicollis (collard flycatcher)	Increased brood size	Reduced resistance to Haemoproteus infections (♀)	Nordling et al. (1998)
Parus major (great tit)	Increased brood size	Reduced resistance to plasmodium (♂; ♀ not affected)	Richner et al. (1995)
Urosaurus ornattus (lizard)	Stimulated vitellogenesis via FSH injections	Reduced wound healing (♀)	French et al. (2007)
Immunological manipulation			
Allonemobius socius (ground cricket)	Injection with LPS	Reduced calling song energetics and nuptial gift size (♂)	Fedorka and Mousseau (2007)
Anopheles gambiae (mosquito)	Injection with LPS	Recuded egg production and yolk protein (♀)	Ahmed et al. (2002)
Gryllus campestris (field cricket)	Injection with LPS	Reduced wing harp size (produces sexual signal) (♂)	Jacot et al. (2005)
Bombus terrestris (bumblebee)	Injection with LPS	Reduced colony success	Moret and Schmid-Hempel (2004)
Passer domesticus (house sparrow)	Injection with Paramyxo virus vacine	Increased clutch size (♀)[a]	Bonneaud et al. (2004)
Ficedula hypoleuca (pied flycatcher)	Injection with tetanus vacine	Reduced fledgling quality and number (♀)	Ilmonen et al. (2000)
Parus caeruleus (blue tit)	Injection with diptheria-tetanus vaccine	Reduced nestling feeding rate (♀)	Raberg et al. (2000)

[a] contrary to trade-off expectation

As stated with the observational approach, an increased pathogen load resulting from increased reproductive effort does not necessarily support a direct causal link, considering that increased effort may lead to increased parasite exposure. To address this, one should also manipulate immune function. In order to evaluate the true trade-off potential, however, researchers must disentangle the costs of immune system deployment from that of an active, detrimental parasite. Accordingly, eco-immunologists have adopted a variety of techniques that allow the partitioning of these affects. In vertebrates, this is commonly achieved with an injection of a non-replicating immune elicitor (e.g. phytohaemagglutinin) and subsequently assessing either the inflammatory response or antibody production (Knowles et al. 2009). In invertebrates a similar effect is achieved through the injection of lipopolysaccharides or peptidoglycan derived from bacterial cell walls (Siva-Jothy et al. 2005). Regardless of the method, immunological manipulation strongly supports the notion of a phenotypic trade-off between reproduction and immunity in both sexes (Table 3.2). This phenomenon is also suggested at the level of gene transcription, where immune challenged *D. melanogaster* transiently suppressed the expression of non-essential metabolic genes (De Gregorio et al. 2001).

It should be noted that some studies have failed to uncover the expected immunological trade-off (e.g. Williams et al. 1999) or have even resulted in a positive association between reproduction and immunity (Shoemaker et al. 2006; Bonneaud et al. 2004; Rolff and Kraaijeveld 2003). With regard to the latter pattern, an acute increase in immune defense after mating seems logical, as various pathogens may be passed during copulation that must be defended against (McGraw et al. 2004; Winterhalter and Fedorka 2009). However, a prolonged increase in immunity following a single mating event seems difficult to reconcile with natural selection because it suggests that pathogen defense prior to mating is less important than pathogen defense after mating. If we assume that a trade-off does indeed exist, however, then several mechanisms may account for the unexpected results.

First, trade-offs might only become evident under stressful conditions (Lazzaro and Little 2009). This appears to be the case in bumblebees where a trade-off between immunity and survival was only apparent after workers were starved prior to treatment (Moret and Schmid-Hempel 2000). McKean et al. (2008) also found that the negative association between bacterial resistance and fecundity in *D. melanogaster* was only present when flies were reared in a resource poor environment. Second, if variation in resource acquisition is greater than variation in resource allocation, than a negative correlation may be obscured or even rendered positive (Van Noordwijk and Dejong 1986; Wilfert et al. 2007). This could occur if variation in environmental quality or access to food were poorly controlled in the experiment, allowing some individuals to acquire more nutrients than others. Third, the type of the immunological assay and the timing of its measurement may dramatically influence the experimental result. Previous work in *D. melanogaster* suggests that increased male sexual activity results in an inferior ability to clear bacterial infections, consistent with an immunological cost to mating (McKean and Nunney 2001). However, later work by another research group suggested that immune genes are actually up-regulated directly after copulation, implying that mated individuals

are more immunocompetent (McGraw et al. 2004). These are clearly contradictory patterns that would have divergent evolutionary implications. As it turns out, mating-induced immune gene up-regulation is transient, lasting approximately 24 hours (Winterhalter and Fedorka 2009). After this time, transcription levels may actually drop below normal, increasing the parasite susceptibility of mated individuals (Fedorka et al. 2007). These studies highlight the importance of assay timing. It also appears that mated individuals are more susceptible to bacterial infections directly after mating despite the fact that this period coincides with increased immune gene transcription (Fedorka et al. 2007; Short and Lazzaro 2010). Thus, increased measures of potential immunity (e.g. immune gene up-regulation) do not necessarily imply increased immune defense (Adamo 2004).

One aspect of immune system trade-offs that is dramatically underexplored is whether certain immunological pathways are more prone to trading-off than others. This question is difficult to answer from an examination of the current literature because most studies do not provide a holistic perspective of the immune system. That is to say, most studies focus on one or two convenient immunological assays, while neglecting various other immune pathways. Some evidence does exist that certain immune pathways are more sensitive to reproduction than others. For instance, genes for humoral antibacterial peptides were significantly modified after mating in *D. melanogaster*, while genes associated with cell-mediated immunity remained unchanged (Winterhalter and Fedorka 2009). Furthermore, the extent to which gene regulation was modified was sex dependent. Thus, immune pathways may be prioritized when trading-off and this prioritization may be sex-specific. It also suggests that the appearance of a trade-off may be somewhat dependent on the immune pathway chosen for study. However, much more work needs to be done before firm conclusions can be drawn.

3.1.3 Mediation of Trade-Offs

Traditionally, biologists have used the Y-model of resource allocation as a descriptive tool to explore the phenomenon of life-history trade-offs. However, the two most important functional components of the Y-model are generally unknown. Specifically, both the product that is being mediated, and the mediator of the product, have often remained intangible model components (Fig. 3.1). Active research over the past several decades, however, has begun to implicate numerous endocrine products in the role of trade-off mediation (e.g. insulin, glucocorticoids, gonadal steroids and melatonin; Harshman and Zera 2007; Ricklefs and Wikelski 2002; Richard et al. 2005; French et al. 2010; Folstad and Karter 1992; Haldar et al. 2012). With regard to the trade-off in mammals, the adipocyte-secreted hormone leptin may play a critical role. Circulating levels of leptin reflect body lipid content in mice, which provides feedback on current condition (Frederich et al. 1995). Furthermore, leptin directly influences t-lymphocyte response to infection. If high reproductive demand reduces adipose, then leptin levels should also drop, which would in turn create an

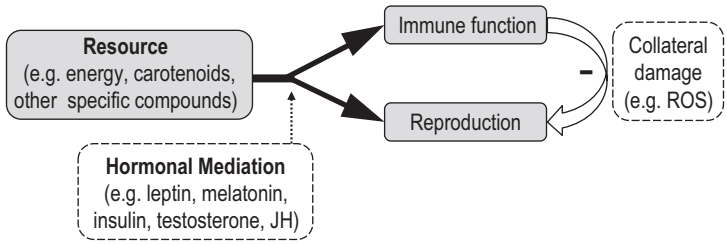

Fig. 3.1 Y-model of resource allocation. The resource being mediated may be energy (e.g. ATP), and/or some other limited compound (e.g. carotenoids). Mediation of the resource into competing pathways is likely control by various endocrine products. Reproduction and immune paths can be further bifurcated into smaller paths within each system. Note that collateral damage due to immune effector deployment may produce a trade-off pattern, but acts independently of shifts in resource allocation

immunosuppressive effect. In support of this model, Lord and colleagues (1998) showed that artificially increased leptin levels could rescue mice from starvation-induced immune-suppression.

Similar hormonal regulation appears to exist in invertebrates. For instance, mating leads to increased juvenile hormone titers, which induces the up-regulation of numerous reproductive pathways (e.g. egg production; Flatt et al. 2005). Work in the mealworm beetle, *Tenebrio molitor*, suggests that juvenile hormone also inhibits a key immune effector system governed by the phenoloxidase enzyme (Rolff and Siva-Jothy 2002). Thus, the bifurcation of energy between reproduction and immunity in invertebrates may be mediated in part by juvenile hormone.

The examples above suggest that the trade-off mediators are hormones, while the product under mediation is energy. Although energy mediation may lie at the heart of numerous trade-offs, both reproductive and immune systems may also directly compete for specific compounds. Perhaps the best example of such a trade-off involves carotenoids. Animals use carotenoids for a variety of biological functions, but are incapable of synthesizing them *de novo*. They are instead acquired from a numerous dietary sources (Horak et al. 2001). These organic pigments are often used in sexual displays, from the orange spots on male guppies (Kolluru et al. 2006), to the bright beaks of male zebra finch (McGraw and Ardia 2003). Furthermore, carotenoids have a positive but complex influence on immune function. Dietary β-carotene provides protection against bladder, kidney and gut infections; stimulates the growth of the thymus gland; increases the number of thymic small lymphocytes; and enhances the cytotoxicity of natural killer cells (see Chew and Park 2004 and references therein). The positive influence carotenoids have on both reproductive and immune physiology, and their classification as an important but limited resource, places them in an ideal position for competitive allocation. As expected, several studies in birds have shown that activation of the immune system indeed results in reduced carotenoid-based sexual signals (Faivre et al. 2003; Baeta et al. 2008).

It is important to note that the Y-model of resource allocation may not always underlie an observed trade-off. Recent studies in vertebrates suggest that sperm viability and motility are compromised when co-incubated *in vitro* with bacteria (Hosseinzadeh et al. 2001; Yaniz et al. 2010; Zan Bar et al. 2008). Therefore, a reduction in male fertility upon bacterial infection may be the direct result of the parasite and not due to competition for a limited resource. As noted in Sect. 8.1.1, part of the cost of immunity may also be due to immunopathology, which is largely resource independent. Immunopathology refers to the collateral damage of somatic and reproductive tissue imparted by the immune effectors deployed during an immune response. In fact, host collateral damage of somatic and reproductive tissue due to continued immune deployment throughout life may play an important part in aging and reproductive senescence (Promislow et al. 2006; Franceschi et al. 2000).

In general, it appears that gametes are susceptible to immune collateral damage, as evidenced by the testes being an immune-privileged site. Spermatozoa are exceedingly susceptible to reactive oxygen species (ROS) in particular, which are both a byproduct of cellular metabolism and an effector component of the innate immune system (Helfenstein et al. 2010; Dowling and Simmons 2009). Thus, the activation of several immune pathways upon infection (e.g. oxidative bursts from phagocytes or ROS byproducts from the phenoloxidase cascade) may have a detrimental effect on reproductive success independent of the resources shuttled towards reproduction (Fig. 3.1). Work on *D. melanogaster* in my laboratory suggests that this is indeed the case (Radhakrishnan and Fedorka 2012). In short, we found that both a live pathogen (*Pseudomonas aeruginosa*) and a pathogen mimic (peptidoglycan) reduced male sperm viability 24 hours after a systemic inoculation. In addition, we found the same pattern of sperm death within the female sperm storage organ when females were similarly inoculated. However, sperm death did not appear to result from a reproductive-immune system trade-off, considering that sperm were able to survive the same duration of time (24 hours) *in vitro* once removed from their somatic resources. These data suggest that the direct interaction between reproductive and immune products may have an underappreciated importance on individual fitness.

3.1.4 Modification of Life Histories to Infection

According to life history theory, populations that experience high rates of extrinsic mortality (e.g. predation) may evolve life history modifications that minimize the mortality cost (Roff 1992; Stearns 1992). In populations of Trinidadian guppies (*Poecilia reticulate)* where the risk of predation is high, an earlier age at first reproduction has evolved (Reznick et al. 1997). Such a dramatic shift in the reproductive schedule, however, was accompanied by several significant costs, including smaller adult body size, smaller eggs, and reduced fecundity (Gordon et al. 2009). As with predation, parasites represent a significant cause of extrinsic mortality and should therefore select for similar changes in life history strategy if the risk of parasitism

is high. Evidence for such an evolutionary response was found in several species of snail, where populations parasitized by castrating trematodes exhibited smaller body size (and likely constrained reproductive success) compared with unparasitized populations (Lafferty 1993; Jokela and Lively 1995). Moreover, parasitism in one snail species (*Potamopyrgus antipodarum)* shaped the population's mode of reproduction by parasitizing parthenogenetic hosts over sexual hosts (obligate sexual and parthenogenetic genotypes coexist in the same population), causing an increase in the sexual genotype (Jokela and Lively 1995). Thus, parasitism selected for sexuality, supporting a basic prediction of the Red Queen hypothesis for the evolution of sex.

Although the above shifts in life histories were evolutionary in nature, a similar plastic response can be achieved within a single parasitized host. In general, if a host's residual reproductive value is low prior to infection, or significantly reduced by the infection, then a host may increase its reproductive effort instead of exhibiting the stereotypical reduction in reproductive effort documented in Table 3.2 (Schmid-Hempel 2011). For instance, if a host is in its juvenile stage upon infection, then it may move its age at first reproduction forward (McCurdy et al. 2001). This response was noted in the female (but not male) mosquito, *Culex pipiens*, which pupated earlier and at a smaller size when infected with the microsporidian *Vavraia cuicis* (Agnew et al. 1999). Alternatively, if the host is already reproductively active, then it may dramatically increase its reproductive effort. Female house crickets (*Acheta domesticus*) exhibited an increased fecundity when infected with the bacterium *Serratia marcesens* (Adamo 1999). However, no life history response was noted when the crickets were exposed to the parasitoid *Omina ochracea*. These strategies are not mutually exclusive, considering that a host may simultaneously accelerate its age at first reproduction and increase its reproductive rate.

A large number of interacting intrinsic and extrinsic factors may influence a host's residual reproductive value, including the severity of the infection, the probability of reproduction (initial or continued) and various environmental variables (Fig. 3.2). Several of these factors may have led to the inconsistencies in the above cited studies. All else being equal, if an existing infection is able to be cleared or tolerated with minimal resources, then one would expect a deceleration strategy (i.e. where the host reduces reproductive activity while increasing investment in immune defense). In contrast, if a host is of advanced age, or is at the end of a fleeting breeding season, then it may be more sensitive to an alternative life history modification (Copeland and Fedorka 2012). This appears to be the case in the blue footed booby (*Sula nebouxii*), where immune challenged younger males decreased their reproductive effort, while immune challenged older males exhibited an increased effort (Velando et al. 2006). Sex may also play in important role, considering the different ways in which males and females maximize fitness. For instance, male fitness is generally constrained by the number of successful (but infrequent) mating attempts, while female fitness is constrained by the rate of offspring production. Thus, males may be more sensitive to modification because of a potentially greater fitness return compared with females.

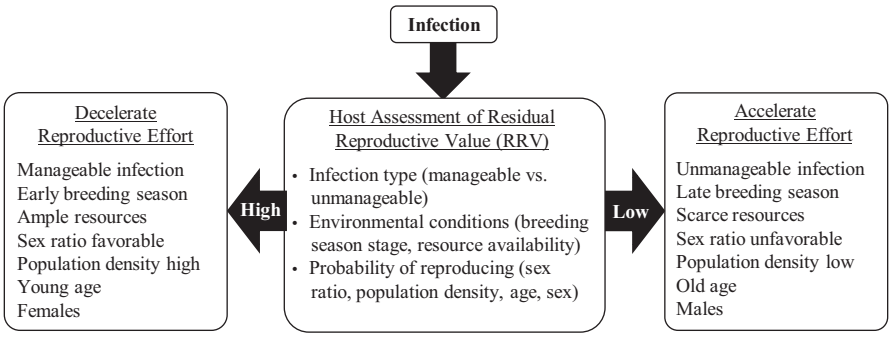

Fig. 3.2 The role of residual reproductive value (RRV) in decelerating or accelerating host reproductive effort upon infection. If RRV is low, then hosts may exhibit and increase in reproductive effort. However, if RRV is high, a deceleration in reproductive effort may occur. Numerous interacting factors (some of which are listed above) may influence RRV, including infection type, environmental conditions, individual condition and the probability of reproducing

Unfortunately, little attention has been paid to the interacting factors that influence a host's life history response to infection. Thus, some of the predictions in Fig. 3.2 are speculative. It should also be remarked that a significant fitness cost (in addition to parasitism) must be paid for accelerating reproductive age or output. Such a life history modification still represents a trade-off, in that the host must extract resources from another physiologically demanding pathway. This may be done at the expense of offspring quality (e.g. reduced egg size or lipid content), host longevity (a.k.a. terminal investment), or some other fitness-related trait.

3.2 Parasite-Mediated Sexual Selection

3.2.1 Genetic Benefits

Until this point, I have focused on the life history trade-off paradigm to highlight how reproductive pathways can indirectly constrain the evolution and/or expression of immune pathways (and *vice versa*). I now turn to how the threat of parasites can directly shape reproductive adaptations through sexual selection. The concept of parasite-mediated sexual selection (PMSS) was introduced by Bill Hamilton and Marlene Zuk (1982) to explain a single, paradoxical observation. In many systems, females appear to choose males in order to obtain a genetic benefit for their offspring. This observation is paradoxical because continued selection for "good genes" should eventually erode all genetic variation in the target of choice (Fisher 1930), yet choice persists (Andersson 1994). To solve this paradox, they proposed that males advertise their heritable ability to resist parasites through showy secondary sexual characteristics. Resistance is based on a matching-alleles model, where a specific match between host and parasite must exist for resistance to be conferred.

Genetic variation is maintained by a host-parasite coevolutionary dynamic that leads to a stable limit cycle in host resistance allele frequencies (as well as parasite exploitation allele frequencies). In other words, resistance allele frequencies ebb and flow over time in response to shifting allele frequencies within a parasite population. Those genotypes that are currently able to resist a common and debilitating parasite can fully invest in their secondary sexual character, honestly advertising their resistance.

The Hamilton-Zuk hypothesis (HZH) has had a profound influence on the study of sexual selection, having been cited over 2000 times since its publication and spawning several other connected avenues of research (e.g. the immunocompetence handicap hypothesis, parasite avoidance hypothesis, fertility assurance hypothesis, and aspects of ecoimmunology). Moreover, it pushed the importance of host-parasite interactions with regard to reproduction to the forefront of evolutionary thought. Thirty years after its publication, however, it remains a controversial model. This controversy stems from an abundance of mixed results (e.g. Read and Harvey 1989), which may in part stem from its seemingly contradictory predictions (Westneat and Birkhead 1998). As a first step in clarifying these issues, it is important to distinguish parasite mediated sexual selection based on genetic benefits from models based on direct benefits. Both models rely on the production of costly honest signals that indicate a parasite-free host. However, signal honesty in the HZH is governed by allelic resistance to parasites, whose variation is maintained by a host-parasite coevolutionary dynamic. In contrast, direct benefit models are governed by the allocation of limited resources or immune collateral damage (i.e. genetic independent) and do not address the maintenance of genetic variation. I discuss the direct benefit models in the next section (Sect. 8.2.2). In Sect. 8.2.3, I then compare and contrast genetic and direct benefit model predictions and present methods that can distinguish between the two.

3.2.2 Direct Benefits

The genetic benefit model is fundamentally different from direct benefit models in that it assumes all individuals are exposed to parasites, but individual variation in parasite resistance exists. The direct benefit models assume that individuals vary in parasite exposure, but all individuals are equal in their resistance. Perhaps the most obvious direct benefit of PMSS is the transmission avoidance hypothesis. Here, female choice is aimed at avoiding sexually transmitted pathogens, which are common in both vertebrate (Sheldon 1993; Lockhart et al. 1996) and invertebrate systems (Knell and Webberley 2004). Sexually transmitted pathogens induce a variety of fitness reducing pathologies in females, such as reduced maternal lifespan, fecundity and offspring viability (Knell and Webberley 2004). As stated in Sect. 8.1.2.2, if a male is suffering from a parasitic infection, he will have fewer resources to devote to his sexual signal. This physiological trade-off can provide females with an honest assessment of transmission risk (Table 3.1). Unfortunately,

definitive tests of the transmission avoidance hypothesis, where females are shown to avoid males infected with sexually transmissible pathogens that influence the sexual signal and female reproductive potential, are lacking.

In addition to transmission avoidance, females may assess the male signal for information on paternal resources. The resource provisioning hypothesis assumes that parasitized males have fewer resources to devote not only to their sexual signal, but to offspring provisioning. This appears to be the case in the cricket *Allonemobius socius*, were females choose males based on the energetics of their calling song (Olvido and Wagner 2004), which is reduced when males are immune activated with lipopolysaccharides (Fedorka and Mousseau 2007). Moreover, immune activation reduces the size of the somatic nuptial gift (hemolymph) provided to the females during copulation, which in turn reduces female reproductive success (Fedorka and Mousseau 2002). It should be noted that a reduction in paternal effort is not limited to a parasitic effect, but may be due to other energetically costly activities unrelated to immune defense.

An extension of the resource provisioning hypothesis states that females utilize the sexual signal to choose males with increased ejaculate quality (Skau and Folstad 2005; Folstad and Skarstein 1997). In infected males, ejaculate quality may be reduced through resource allocation or through immune collateral damage (Sect. 8.1.3; Fig. 3.1). Support for this hypothesis stems from the general observation that bacterial and viral infection is associated with reduced sperm number, motility and viability (Al-Qarawi et al. 2004; Boltz et al. 2007; Lorusso et al. 2010; Liljedal et al. 1999). Direct systemic immune challenges with both live pathogens and non-replicating immune elicitors provide similar results (Simmons 2012; Radhakrishnan and Fedorka 2012). Furthermore, the reduction in sperm quality due to oxidative stress (which can be an immune response by product) in the great tit, *Parus major*, appears to be buffered best by brightly colored males (Helfenstein et al. 2010). However, no one study to date has simultaneously assessed all predictions of the fertility assurance model; specifically, that parasitic infection leads to a reduction in sperm quality, male sexual signal, female choice and female reproductive potential.

3.2.3 Comparison of Genetic and Direct Benefits Models

Intraspecific investigations into the HZH generally predict a negative phenotypic correlation between the sexual signal and parasite load (Fig. 3.3a, model *t*). Parasite load refers to the current abundance of parasites infecting a host, and is assumed to be a proxy for parasite resistance. Parasite resistance is defined here as the ability to efficiently defend against parasitic infection (i.e. the most resistant individuals require the fewest resources for immune defense). According to the HZH, resistance is genetically based (Hamilton and Zuk 1982). As mentioned earlier, the HZH has endured mixed results. In particular, intraspecific studies across various vertebrate taxa have had great difficulty supporting its predictions (Hamilton and

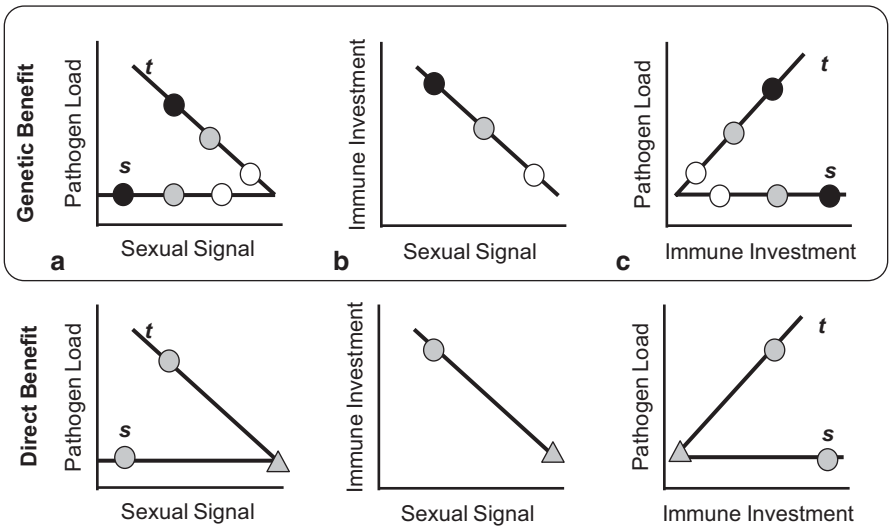

Fig. 3.3 **a**–**c** Comparison of phenotypic predictions for both the genetic benefit model (GBM) and direct benefit models (DBM). Both models rely on the production of a condition-dependent honest signal that indicates a parasite-free host, and assume that all genotypes are equivalent in their capacity for resource allocation and acquisition. In both models, individuals may exhibit two different response strategies to infection, including sterilization (*s*) and tolerance (*t*). Importantly, the GBM assumes that parasite exposure is ubiquitous, but resistance is variable, while the DBM assumes that parasite exposure is variable, but degree of resistance is ubiquitous. For all graphs, *circles* and *triangles* represent infected and uninfected individuals, respectively; *black*, *gray* and *white* shapes indicate susceptible, moderately susceptible and resistant genotypes. Column **a**: The GBM predicts a negative relationship between pathogen load and signal under the tolerance strategy, with the most resistant genotype exhibiting the greatest signal and lowest pathogen load. For the sterilize strategy, a neutral relationship is expected. If heritable parasite resistance that is manifest through the signal exists, then model *s* would result in Type II error. Where the two strategies intersect in the bottom right corner represents the population average expression of the signal prior to infection. The same phenotypic relationship is expected for the DBM. Column **b**: Regardless of the immune response strategy or benefit model, a negative phenotypic relationship is expected between immune investment and signal. Column **c**: Implicit in first two relationships, is the relationship between immune investment and pathogen load. The slop of the model depends on the response strategy. Again, both models make similar predictions. See text for all assumptions

Poulin 1997). Two important points must be addressed regarding the common intraspecific predictions that dramatically alter their interpretation.

First, it may be possible for individuals within a population to either completely clear the parasite (sterilization), or strategically manage some level of infection (tolerance). The strategy adopted may depend on parasite virulence, which can be either plastic or fixed in origin (Boots et al. 2009). In accordance with the HZH, the cost of both strategies would be paid by allocating resources away from the condition dependent signal. However, the cost for the resistant genotype will always be less than for the susceptible genotype within a given strategy, and the tolerance strategy requires fewer immune resources than sterilization for a given parasite.

Importantly, the sterilization strategy will result in a neutral relationship between signal and pathogen load even if a population exhibits heritable parasite resistance that is manifest through the sexual signal, leading to Type II error (failure to reject the null hypothesis; Fig. 3.3a, model *s*). In other words, a neutral phenotypic relationship is not necessarily evidence against the HZH. Second, both benefit models predict the same relationship, hampering support for HZH even if a negative relationship were found.

Implicit in the HZH is the underlying relationship between immune investment and the sexual signal. Here, I define immune investment as the abundance of specific immune effectors observed in response to infection. Upon infection, the susceptible genotype will greatly increase their immune investment relative to the resistant genotype. Again, resources for immune defense are expected to be taken from the sexual signal (Fig. 3.3b). Regardless of the pattern between the signal and parasite load in Fig. 3.3a (i.e. negative or neutral) or the response strategy (sterilize or tolerate), the relationship between immune investment and signal is always expected to be negative. Also implicit in the HZH is the relationship between parasite load and immune investment (Fig. 3.3c), which can be either positive or neutral depending on the relationship in Fig. 3.3a. Again, both models predict the same relationships. In short, the phenotypic correlation between immune investment and the signal provides partial support for PMSS, but none of these relationships can differentiate direct from genetic benefit models.

There are, however, explicit predictions that allow the models to be differentiated. The HZH predicts that parasite resistance is heritable and expressed through the sexual signal. Direct benefit models make no such prediction regarding heritability. It is important to note that the sexual signal need not be heritable, only condition dependent (Folstad and Karter 1992; Hamilton and Zuk 1982). Furthermore, the model stipulates that the parasites associated with the target for female choice (resistance) are those engaged in a coevolutionary dynamic with the host. Therefore, testing parasite resistance using a generic pathogen, an artificial immune elicitor, or general circulating immune effectors will not likely provide an accurate assessment of the model.

Assuming that an appropriate pathogen has been identified, one can then test for heritable resistance by inoculating males within a quantitative genetic framework. The simplest result in support of the HZH would be a negative genetic correlation between the sexual signal after infection and resulting pathogen load (Fig. 3.4b, model *t*), where the parasite induces a chronic infection. As stated earlier, a neutral relationship may result if individuals opt to sterilize the infection. If no variation in pathogen load exists (Fig. 3.4b, model *s*), then one could regress the change in the sexual signal upon infection ($\Delta\text{signal} = \text{signal}_{\text{Prior}} - \text{signal}_{\text{post}}$) against the expression of the signal post infection ($\text{signal}_{\text{post}}$; Fig. 3.4c) to avoid the possibility of Type II error. This analysis allows one to assess the genetic variation in the parasite's influence on the sexual signal, providing a definitive test for the HZH. Importantly, direct benefit models do not exhibit the same predictions. No relationship is expected between the signal and pathogen load within strategies (Fig. 3.4b). If both strategies

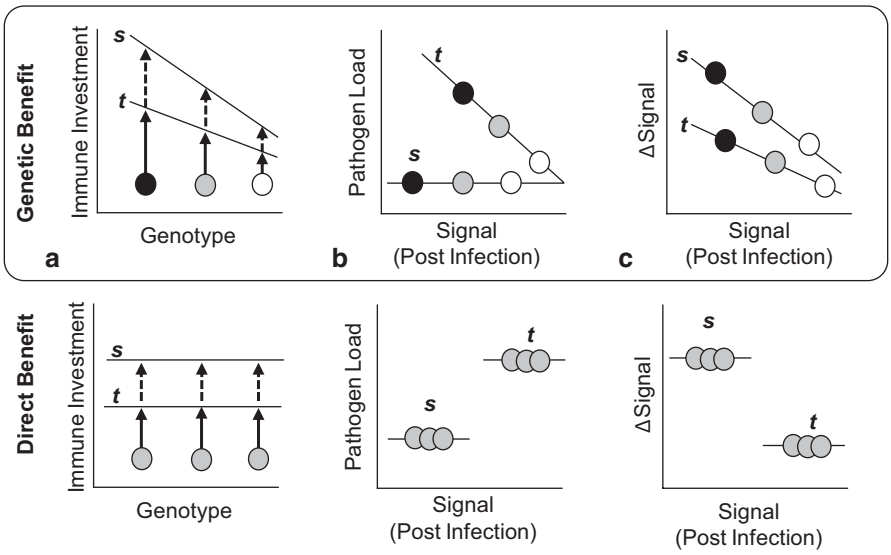

Fig. 3.4 **a**–**c** Comparison of genetic predictions for both the genetic model (GBM) and direct benefit models (DBM). Both models rely on the production of a condition-dependent honest signal that indicates a parasite-free host, and assume that all genotypes are equivalent in their capacity for resource allocation and acquisition. In both models, individuals may exhibit two different response strategies to infection, including sterilization (*s*) and tolerance (*t*). The sterilization response is assumed to require more resources than tolerance. Column **a**: Upon infection with a standardized parasite challenge, the GBM predicts that susceptible genotypes will exhibit the greatest immune investment (*black*, *gray* and *white circles* represent susceptible, moderately susceptible and resistant genotypes, respectively). In contrast, no difference between genotypes in their immune investment is expected under the DBM. Column **b**: The GBM is clearly supported under the tolerance strategy by a negative genetic correlation between signal expression post infection and pathogen load. However, sterilization may lead to a neutral relationship and Type II error. The DBM predicts a neutral relationship between pathogen load and signal for both response strategies. If both strategies are simultaneously employed, a positive relationship would result. If a positive trend is obtained, then the change in sexual signal (Δ Signal) need not be calculated. Column **c**: If hosts engage in sterilizing immunity, one can assess the GBM by regressing the change in the sexual signal (Δ Signal) against the expression of the signal post infection. Both sterilization and tolerance will result in a negative genetic relationship. Again, the DBM predicts a neutral relationship between these variables with a strategy. See text for all assumptions

are simultaneously employed, then a positive relationship would result, indicating that the genetic benefit model is not appropriate.

One additional issue with previous tests of the HZH should be addressed. The HZH predicts a positive relationship between the sexual signal and parasite resistance. Considering that the parasite(s) undergoing the coevolutionary dynamic is often unknown, researchers have used general immune investment as a proxy for parasite resistant, and have reported positive correlations between signal and general immune investment as support of the HZH (e.g. Ryder and Siva-Jothy 2000). However, variation in general immune investment is not equivalent to resistance

to a specific parasite. Furthermore, a positive relationship implies that some hosts have more resources to invest in both the sexual signal and immune function than other hosts, which is likely the product of genetic variation in resource acquisition, not parasite resistance. Although this variation can be maintained via regular cycles in environmental resource abundance, it cannot lead to a host-parasite coevolutionary dynamic in resistance alleles. It stands as a separate model of genetic benefits that has a non-frequency dependent mechanism for the maintenance of genetic variation.

Perhaps the most succinct test of the HZH can be performed at the macroevolutionary level. Hamilton and Zuk (1982) proposed that the intraspecific coevolutionary dynamic would lead to an interspecific positive correlation between species-level signal intensity (i.e. showiness) and parasite load intensity. In other words, the more prone a species is to parasitism, the greater the selection for intense sexual signals. To date, there has been strong general support for this prediction in birds; although, support in other vertebrates systems is weak (Hamilton and Poulin 1997). However, direct benefit mechanisms can also produce the interspecific pattern, as choice for non-parasitized mates (in order to gain direct benefits) will still select for exaggerated signals if the threat of infection is high. Perhaps the most holistic approach in addressing the HZH would be to identify those species at the signal extreme of the interspecific correlation and then address the intraspecific predictions of the hypothesis. The realization that both benefit models produce similar phenotypic patterns within and across species exposes the underlying difficulties in testing the HZH, and the need for more work to resolve the lingering controversy. Hopefully, the discussion and methods presented here provides a clear path forward.

3.3 Closing Thoughts

Throughout this chapter, I have highlighted how the reproductive and immune systems are intertwined in both their expression and evolution. Although we have gained ample ground in understanding the implications of this interaction over the preceding decades, more work remains to be done. For instance, we know relatively little about how genetic architecture constrains the evolution of reproductive and immune traits under natural settings. We should expand our investigations beyond the traditional model organisms reared under selectively benign laboratory conditions and begin to tackle the estimation of pleiotropy in natural habitats. Without this knowledge, we cannot accurately assess the degree to which reproductive selection shapes pathogen resistance in the wild. In addition, we know little about which immune pathways are most sensitive to reproductive trade-offs. It is likely that different immune components are prioritized during the reallocation of resources. Unfortunately, this remains an underexplored avenue of research.

More work is also needed in clarifying the components of the Y-model of resource allocation. Specifically, the mediators (endocrine or non-endocrine) that play key roles in shuttling resources between competing pathways needs continued exploration, as do

the products being mediated (e.g. carotenoids).The extent to which the observed trade-off is influenced by immunopathology also needs to be addressed. This is an exciting area of research as it provides a resource-independent pathway by which immune defense may limit reproductive potential. One final aspect of the reproductive immune trade-off that warrants further exploration is the influence of residual reproductive value in modifying life history response to infection. This requires a close look at numerous interacting biotic and abiotic factors that determine whether a host reduces or accelerates its reproductive effort upon infection.

Our growing understanding of how reproductive effort antagonistically influences immune function has also forced a reassessment of the most studied genetic benefit model of parasite-mediated sexual selection. Here I've shown that traditional tests of the Hamilton-Zuk hypothesis (i.e. phenotypic correlations between sexual signal and parasite load) cannot differentiate it from the more recently derived direct benefit models. Furthermore, I've shown that the integration of different infection outcomes into the model (i.e. sterilize versus tolerance) can lead to Type II error, calling into question the interpretation of both the positive and negative results previously reported. To ameliorate this hazard, I have presented a novel method for assessing the host's response to standardized infection within a quantitative genetic framework that is based purely on the expression of the sexual signal. Hopefully, this approach will help to clarify future tests of the Hamilton-Zuk hypothesis.

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Chapter 4
Host-Parasite Interactions

Jeb P. Owen and Dana M. Hawley

Abstract The immune system represents a complicated assemblage of coordinated genes, proteins, cells and tissues. The field of ecological immunology (EI) is founded on the assumption that immunological defenses incur costs (energetic or fitness) under different ecological conditions. These costs are expected to shape investment in immunity and to alter the dynamics of infection. Considering that the primary function of the immune system is to defend the host against infection, it is warranted to ask—to what extent is immune function meaningful outside of parasitism? Parasites provide a physiological context for immune function. The full complexity of the immune response may only be elucidated by the interplay between diverse tissues, cells and molecules of the host and the parasite. Parasites also provide a conceptual keystone for ecological and evolutionary exploration of immune function. We illustrate these points by describing the connections between immune responses at two scales (cell and tissue) against two taxonomic groups of parasites (unicellular and multicellular). We discuss four challenges for future research in EI: (I) Researchers need to empirically demonstrate host-parasite interactions that affect fitness of the organisms under study; (II) Researchers should interpret immunological traits relative to both defense and tolerance; (III) Tests of immunological traits should include co-infections; (IV) The perspective of the parasite should be more thoroughly considered. We believe addressing these challenges will strengthen the integration of immunology and ecology as the field continues to grow.

Keywords Coadaptation · Disease · Ecology · Immunology · Parasite · Ecological Immunology

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4.1 What is Host-parasite Interaction?

In an ecological context, host-parasite interactions are characterized by the relative fitness (e.g., survival or reproduction) of each interacting organism. A parasitic interaction is of benefit to the parasite, but results in negative fitness consequences for the host. In practice, the extent to which hosts are negatively affected by parasites varies enormously, with some host-parasite relationships perhaps best characterized as commensalisms. The extent of fitness costs to the host are also strongly mediated by the environment, such that a parasite can have no fitness consequences for a host under ideal ecological conditions, but survival or reproductive costs may be apparent following environmental stressors such as extreme weather or food limitation (e.g., Gulland 1992; Howe 1992).

Host-parasite interactions can also be viewed from a physiological perspective, characterized by the molecular and cellular interplay that occurs when parasites encounter host tissues. In some cases, these interactions directly result in host pathology, or negative fitness consequences, via parasite-induced tissue damage, the use of host resources, or direct suppression of host immune responses by the parasite (Schmid-Hempel 2011). In other cases, the negative fitness consequences of parasites on hosts result largely from host-mediated responses to parasitism such as immunopathology, or changes in host behavior that may limit reproductive success (Schmid-Hempel 2011). Whether the negative fitness effects of parasitism are pathogen- versus host-mediated has important consequences for the ecology and evolution of the host immune response (see Immune Strategies Section Below).

4.1.1 The "History" of Ecological Immunology and Parasites

The field of ecological immunology (EI) is a relatively young but rapidly expanding discipline (Martin et al. 2011). This field was founded on the assumption that immune defenses incur some type of cost (opportunity, physiological, evolutionary, etc.), and therefore an individual's investment in immunity is expected to vary with any intrinsic and extrinsic factors that alter the relative costs and benefits of immune investment. This basic assumption led to an explosion in research efforts to identify the ecological and evolutionary factors that shape immune function over time and space (Martin et al. 2011). Although these studies have contributed enormously to our understanding of many fields, including life-history trade-offs, behavioral ecology, and sexual selection, the majority of EI studies have evaluated immune function independent of parasites or pathogens (Owen et al. 2010; Hawley and Altizer 2011). Considering that the primary function of the immune system is to defend the host against infection, it is warranted to ask—to what extent is immune function meaningful outside the context of parasites and pathogens?

In this chapter, we explore the growing intersection of ecological immunology and host-parasite interactions by first describing the complex interface between parasites and the host immune system. We then discuss what is currently missing from

the EI field with respect to parasites and what can be gained by both fields (EI and parasitology) by conducting work at the intersection of host-parasite interactions and ecological immunology. We conclude with some of the challenges and considerations inherent in incorporating parasites into ecological immunology studies.

4.2 Interface Between the Parasite and the Immune System

The immune system represents one of the most complicated assemblages of coordinated genes, proteins, cells and tissues that operate in higher organisms (Wodarz 2006). Although the immune system plays a key role in the regulation of cancerous cells and is responsive to foreign substances from the environment (e.g. allergens), it is generally accepted that the central role of the immune system is to defend the organism against infection by pathogens and parasites* (Parslow et al. 2001).

*[For the purpose of this discussion, the term parasite will include microscopic "pathogens"—e.g. viruses, bacteria, protozoa, fungi—and macroscopic "parasites"—e.g. worms and arthropods.]

A brief glance at any introductory textbook on vertebrate immune function quickly reveals the remarkable complexity of genes, proteins, cells and tissues that are involved in immune defense (see Box 1). It is far beyond the scope of this chapter to comprehensively review the components of immune defenses that occur among animals. Instead, we direct the reader to two excellent texts on immunology (Parslow et al. 2001; Janeway et al. 2005). Relative to vertebrates, the understanding of the invertebrate immune system is still shallow. However, research continues to accumulate that reveals the invertebrate immune system also reflects diverse cells and proteins that produce a dynamic defensive response against parasites (see: Castillo 2011; Gonzáles-Santoyo and Córdoba-Aguila 2012; Hancock 2006; Stanley 2009). Finally, although this chapter focuses on the ecological immunology of animals and their parasites, the reader is reminded that plants also have inducible physiological defenses against parasites, which have been considered in the context of ecological immunology (see: Alcazar et al. 2011; Baucom and de Roode 2011). In place of a generic review of vertebrate immune function, we present a simplified diagrammatic summary of the interactions among compartments of the immune system that are involved in responses to different taxa of parasites (see Box 2).

There are numerous ways to conceptually divide a discussion of host-parasite immunological interaction. Classically, immunologists separate innate versus adaptive responses and discuss the involvement of cellular versus humoral immune effectors. Parasitologists often categorize interactions by parasite taxonomic units (e.g. protozoa versus metazoa), or by the spatial location in the host (e.g. intracellular versus extracellular). These distinctions undermine an important commonality across most host-parasite relationships—the reciprocal interactions between the host immune system and the parasite engage both parties at multiple scales and involve diverse molecules, tissues and cell types. Here we attempt to illustrate this

complex integration by drawing attention to the connections between immune responses at two scales (cell and tissue) against two taxonomic groups of parasites (unicellular and multicellular). These combinations of scale and parasite biology invoke unique and illustrative attributes of host immune defense. However, they also share a multitude of linkages in the immune response, broadly, which may represent the most relevant connections between immune function, parasitism and ecology.

4.2.1 Example 1: Intracellular, Protozoan Parasite

Protozoa in the genus *Leishmania* are intracellular parasites of phagocytic and lymphoid cells in the vertebrate host. These parasites use diverse vertebrates as hosts (e.g. dogs and rodents), often without obvious pathological effects. In contrast, *Leishmania spp.* cause severe forms of infectious disease in humans across the tropics/subtropics worldwide. For example, *L. braziliensis* in Central and South America causes a muco-cutaneous form of infection that results in the devastating erosion of tissue in the palate and sinuses. *L. donovani sp.* in China, India and parts of Africa infect cells in the liver, spleen and mesenteric lymph nodes, which left untreated is fatal after 2–3 years. The *Leishmania* protozoa are transmitted between vertebrate hosts by blood-feeding flies ("sandflies") in the subfamily Phlebotominae (Roberts and Janovy 2000).

After injection into the host by the fly, the *Leishmania* invade phagocytic macrophages and neutrophils that respond to the blood-feeding ectoparasite (Peters et al. 2008; Ritter et al. 2009). This represents the first step in the interaction between the host immune response (innate) and the parasite. The ability of the host to limit, or stop infection at this point relies on cell signaling typically linked with innate immunity (e.g. Toll-like receptor activation) (Blackwell 1985; Gallego et al. 2011). Subsequent control of the parasite requires involvement of the adaptive immune response (e.g. CD4+ helper T lymphocytes) (Gollob et al. 2005), which requires a bridge between innate triggers (e.g. complement proteins) of phagocytic cells and adaptive processes (e.g. antigen presentation to lymphocytes via MHC expression) (Blackwell et al. 1985; Aebischer et al. 2005). Thus, the involvement of the immune response, spanning from cell invasion to pathogen isolation/clearance, requires the involvement and cross-talk of multiple proteins (complement, immunoglobulin, toll-like receptors) and cell types (macrophages, dendritic cells, neutrophils, lymphocytes) from both the innate and adaptive arms of the immune system.

Further complicating this system of immune responses is the role of the sandfly. Sandfly saliva contains potent vaso-dilating and immunomodulating molecules that strongly affect the immunological environment of the skin where the *Leishmania* is introduced (Brodie et al. 2007; de Moura et al. 2010). Host immune responses against the sandfly negatively affect sandfly fitness and reduce transmission of *Leishmania* (Morris et al. 2001). These observations underscore the complexities of the immune response and the linkages across "arms" of the immune system.

4.2.2 Example 2: Extracellular, Metazoan Parasite

Flatworms (Trematoda) in the genus *Fasciola* are parasites that undergo asexual replication in lymnaeid snails and sexual replication in mammals (e.g. sheep, cattle, goats, elk, elephants, pigs and humans). In the mammalian host, the parasites settle in the bile ducts of the liver, where they shed eggs into the intestine to be passed out of the host with feces. The parasites cause inflammation in the bile ducts that leads to scarring, atrophy of the liver parenchyma and cirrhosis (Robert and Janovy 2000). Two species, *F. hepatica* and *F. gigantica* are serious economic pests of sheep and cattle, worldwide, and can parasitize humans (Piedrafita et al. 2010; Pleasance et al. 2011).

The infective stage for mammals encysts on vegetation, or in the water, and is ingested by the host animal. The parasite excysts in the small intestine, penetrates the intestine wall and migrates through the coelom to the liver. During migration through these tissues, the parasite stimulates immune responses from the host. In Indonesian thin-tail (ITT) sheep, macrophages from the peritoneal cavity are capable of killing juvenile *F. gigantica*. Following repeated exposures, ITT sheep produce an antibody-mediated (adaptive) immune response that directs macrophages and eosinophils against the juvenile parasites, which blocks successful infection (Piedrafita et al. 2007). Once in the liver, *Fasciola* migrates to the bile ducts, where it completes sexual maturation. In the liver of cattle and buffaloes, T- and B-lymphocytes, eosinophils and mast cells are involved in the local inflammatory response to *F. gigantica*. Interestingly, the local T-cell response in the liver appears to decrease over the course of infection in cattle, which are susceptible to *F. gigantica,* but increases in the liver of swamp buffaloes, which are resistant (Molina and Skerratt 2005). Successfully established *Fasciola* shed eggs into the bile ducts, where they are carried out to the intestine and expelled into the environment with the host feces. The eggs, which are not infectious for mammals, also stimulate an immune response. Cattle produce immunoglobulins that bind to *F. hepatica* egg-associated proteins and circulating immune cells exposed to those proteins will produce pro-inflammatory cytokines (Moxon et al. 2010). Though the impacts of the immune responses to *F. hepatica* eggs are currently unknown, these data illustrate how the host immune system is responsive to the parasite through multiple stages of development and across multiple types of tissue.

Finally, it is important to note that snails also produce immune responses to infection by trematodes. Experimental infections of the snail *Biomphalaria glabrata* with two trematode species produced responses from genes involved with recognition/binding of foreign proteins and phagocytosis (Hanington et al. 2010). Among lymnaeid snail species, these types of immunological responses may be associated with pronounced differences in susceptibility to infection by *Fasciola sp.*(Calienes et al. 2004; Bargues et al. 2011). Thus, the relevance of host immune function to host-parasite interaction can extend to multiple host taxa through the parasite life cycle.

In summary, parasites engage the immune system through multiple pathways that include molecular and cellular responses to tissue damage, as well as recognition of parasite-associated molecular patterns. These pathways represent both the classical

innate and adaptive elements of the immune system, which typically act in concert. As illustrated by the examples above, "simple" (unicellular) and "complex" (multicellular) parasites will both elicit multifaceted, dynamic responses from the immune system. Importantly, no single pathway in the immune response is responsible for host defense. This reality may have the unfortunate effect of leading researchers in EI to conclude that host-parasite interactions are too complicated to tackle. Alternatively, we hope that these complexities have the inspiring effect of showing the many fascinating ways that immune function could intersect with host ecology and host-parasite interaction.

4.3 Parasites in EI: What is Lacking and What can be Gained?

As discussed in the introduction, parasites are often overlooked, or excluded from studies in EI. In the following section, we address what is lacking in EI studies as a result and what can be gained for both fields by bringing parasites into the EI framework of research.

4.3.1 Demonstrating Host-parasite Interaction

An implicit assumption in both EI and parasitology is that host immunological traits are linked to host fitness. Nonetheless, a facet often missing from EI is evidence that host-parasite interactions involve host immune function and affect host fitness. Most students of parasitology and infectious disease are taught "Koch's Postulates", which provided the first empirical framework for determining a causal relationship between a suspected pathogenic organism and an infectious disease (Koch 1884). The original postulates have been amended and expanded to capture nuances of infectious disease, such as the asymptomatic carriers of a pathogen (Inglis 2007). However, the central purpose of the postulates remains the same—to provide definitive evidence that a parasite and host interact to affect host health (fitness). For researchers in EI, there are often significant obstacles to obtaining definitive evidence for interaction between parasites and hosts.

First, many EI study systems involve wild animal populations where the parasites may be unknown. If the suite of parasites is unknown for a subject species, it may be experimentally difficult to identify them and subsequently test each species for an effect on host fitness. Moreover, most animals (especially wild species) are co-infected with multiple taxa of parasites that may confound experimental efforts to link immune function, parasite resistance and host fitness (see Box 2, and Co-infection, below). Second, pathogenic effects of parasites may be transient, or context dependent. These complexities can make it challenging to design EI experiments that will provide conclusive tests of hypotheses. For example, pathogen transmission may be highly seasonal (e.g. Mycoplasmal conjunctivitis in House finches; Altizer et al. 2004), or sporadic (e.g. Plague in Prairie dogs; Savage et al. 2011),

such that the time frame for host-parasite interaction is brief and difficult to access experimentally. Pathogen susceptibility and the pathology of infection may also vary among species (e.g. West Nile virus in birds of North America), such that insights from the study of any single species may not apply to other species (Komar et al. 2003; LaDeau et al. 2007).

Though these obstacles exist, there is vital information that can be gained from incorporating host-parasite interaction into EI that should inspire researchers to overcome these admittedly daunting barriers. First, parasites provide a physiological context for immune function. The full complexity of the immune response may only be elucidated by the dynamic interplay between the diverse tissues, cells and molecules of the host and parasite. Second, parasites provide an ecological/evolutionary context for immune function. As discussed in the introduction, the presumptive function of the immune system is to mitigate fitness costs to the host that are imposed by parasites. Thus, understanding the causes and consequences of immunological variation will be most meaningful relative to host-parasite interaction.

4.3.2 Immune Strategies: What is Optimal?

There is a growing appreciation for the diversity of strategies that the immune system may use in order to mitigate the fitness costs of parasite infection (Graham et al. 2011). The field of ecological immunology has historically assumed the "more is better" paradigm, with stronger immune responses representing optimal immune functioning and highest fitness in response to infection (e.g., Nunn et al. 2000). However, it is increasingly appreciated in EI that the optimal or most fit immune responses are often not the strongest (Råberg and Stjernman 2003), and optimality is best measured by functional fitness outcomes rather than the strength of an immune response (Viney et al. 2005; Graham et al. 2011).

There are two important reasons why stronger immune responses are not always better (Viney et al. 2005). First, the costs associated with immune responses, including immunopathology (Graham 2002; Graham et al. 2005), may favor responses that are intermediate in strength (Graham et al. 2010). For example, in a recent study on Soay sheep, the presence of self-reactive antibodies was associated with reduced reproductive success in both sexes, but increased survival in females during years of winter population crashes, suggesting that complex fitness trade-offs are likely associated with immune variation in natural populations. In the Soay sheep system, intermediate immune responses may be favored due to the distinct directions of selection on reproduction versus survival. Unfortunately, studies that relate the strength of immune responses to fitness outcomes in free-living populations remain rare (but see Råberg and Stjernman 2003) due to the difficulties in obtaining long-term fitness measures for most free-living animals. Significantly more studies are needed before any broader conclusions can be drawn regarding the costs and benefits of immune strength in natural populations. Furthermore, immune optimality will depend on the prevalence and burden of parasites present (Tschirren and Richner 2006), whereby strong immune responses may only be selected for in

years or in populations where parasite prevalence is high. Thus, an understanding of parasite dynamics and its influence on host immune optimality are critical in order to understand variation in host immunity over space and time, the purview of EI.

Second, the costs associated with enduring or tolerating a parasite infection may in many cases be lower than the costs associated with mounting the immune response needed to eliminate that parasite (Medley 2002; Viney et al. 2005). Therefore, the cost-benefit analysis for a host will not always favor the rapid clearance of parasite infection (aka, resistance), such that hosts with the lowest parasite numbers are not always the most fit (Behnke et al. 1992; Stjernman et al. 2008). There is a growing appreciation that in many cases, hosts best mitigate the fitness costs of infection by practicing tolerance, or the minimization of fitness cost per unit parasite (Jokela et al. 2000; Rausher 2001; Råberg et al. 2007, 2009; Baucom and de Roode 2011). The immune mechanisms that underlie host tolerance are not well understood, but the balance between pro- and anti-inflammatory signaling is one proposed axis along which the immune system may show variation in tolerance, with more tolerant individuals tipping the balance toward anti-inflammatory responses (Sears et al. 2011). This strategy, while not eliminating the parasite, would minimize costs of parasite infection by suppressing the potentially costly components of the host immune response- in this case, inflammatory processes.

The role of tolerance versus resistance strategies, which are not mutually exclusive, in responding to parasite and pathogen infection have rarely been examined in the context of EI (Baucom and de Roode 2011). Genetic variation in tolerance among populations has been detected in dace fish in response to an ectoparasite (Blanchet et al. 2010), and tolerance also appeared to have an environmental component in this system. Ayres and Schneider (2009) found that food restriction increased tolerance of fruit flies to *Salmonella typhimurium* but decreased resistance to *Listeria monocytogenes*. The results of this study highlight two important points. First, eco-immune studies typically measure parameters more relevant for resistance (aka strength of the immune response), and thus an eco-immune study on fruit flies and food restriction would conclude that fruit flies are more susceptible to pathogen infection under food restriction. This conclusion would miss the important result that tolerance increased under food restriction. Second, the differing host responses to two bacterial pathogens under the same ecological stressor (in this case, food restriction) illustrates the importance of understanding specific host-parasite interactions in order to draw conclusions about ecological stressors and host immune trade-offs. Overall, a detailed examination of parasite dynamics is critical in order to understand the fitness trade-offs that hosts face when practicing either strategy, and whether those trade-offs vary in response to environmental constraints.

4.3.3 Coinfection Dynamics

The fields of EI and parasitology have both been dominated by reductionist approaches, which study immune responses and/or parasites in relative isolation. While reductionist approaches are both necessary and powerful, hosts are a landscape for

diverse communities of parasites that interact both with each other and with the host immune response (Box 2; Petney and Andrews 1998; Pedersen and Fenton 2007). There is now a growing understanding that parasite communities may drive immune system trade-offs and costs within hosts (Koskella et al. 2012), and in turn, that the immune system may be an important driver of the stability and diversity of within-host parasite communities (Ulrich and Schmid-Hempel 2012).

The dynamics of parasite communities within hosts are thought to be driven by both bottom-up and top-down mechanisms (Pedersen and Fenton 2007). From the bottom up, the presence of parasites that compete for similar resources within hosts may result in competitive interactions and, in some cases, competitive exclusion. The host immune system (akin to a "predator") can also act as a strong top-down regulator of parasite communities, either preventing or facilitating persistence of diverse parasites within hosts. Using a meta-analysis of helminth-microparasite coinfecton studies in mice, Graham (2008) found evidence for both types of control in predicting the outcome of coinfection. Immune-mediated interactions between helminths and microparasites have been widely documented in humans, lab animals, and wildlife (e.g., Bazzone et al. 2008; Brown et al. 2006; Ezenwa et al. 2010; Hartgers and Yazdanbakhsh 2006; Secor 2006), in part because helminths modulate the vertebrate immune response in ways that are likely to have strong effects on the outcome of microparasite infections. First, helminth infections tend to bias the T-helper cell response toward a Th2 profile, including the upregulation of Th2-type cytokines and the downregulation of Th-1 cytokines typically involved in fighting off many microparasites (Moreau and Chauvin 2010). Second, helminths are known to cause immunomodulation in hosts via alteration of regulatory T-cell activity, leading to a generalized suppression of both Th-1 and Th-2 responses (Maizels et al. 2004). Both immune-mediated pathways can alter the likelihood or severity of microparasite infections in hosts previously infected by helminths. Furthermore, the nature and extent of these immune-mediated interactions have important consequences for the population-level dynamics of microparasites (Ezenwa and Jolles 2011).

Helminth-microparasite interactions are currently the best-studied class of coinfection due to the clear immune interactions between these two types of parasites and the prevalence of their co-occurrence in humans. However, there are also interactions between other taxa of parasites co-infecting a host, including ecto- and endo-parasites. For example, mice infected with the mite *Myocoptes musculinus* and later infected with *Toxoplasma gondii* show a higher mortality rate, more severe toxoplasmosis, and higher levels of tissue parasitism than mice only infected with *T. gondii*, presumably due to the Th-2 immune response induced by *M. musculinus* infestation (Welter et al. 2007). Ectoparasites that serve as vectors can also alter host immune responses in surprising ways. Prior exposure to uninfected mosquito bites in mice later exposed to West Nile virus caused higher levels of viral replication and altered immune expression (Schneider et al. 2007). Because this effect was mimicked by the passive transfer of mosquito-sensitized serum from mice, the authors conclude that this effect is mediated by host responses to uninfected mosquito saliva. This fascinating example implies that host immune responses to ectoparasites alone can alter the course of infection with the parasites that those ectoparasites

may transmit. Overall, we still have relatively little understanding of the multitude of interactions that may occur within co-infected hosts, but these studies suggest that parasites cannot be viewed in isolation in order to understand the outcome of infection. It is therefore critical for the fields of EI and parasitology to continue to expand beyond the historical reductionist approach in order to characterize potential immune-mediated interactions between co-infecting parasites and the co-infecting parasites and population-level infection dynamics (Ezenwa and Jolles 2011).

The role of the host immune system in facilitating or reducing parasite diversity within hosts is an important contribution that EI can make to a broader understanding of parasitology. Ulrich and Schmid-Hempel (2012) recently showed that trypanosome parasite strains exhibit consistent competitive exclusion in the absence of a host, but *in vivo* the host immune system altered the outcome of competition among strains and facilitated higher rates of co-infection in hosts. The immune system can also reduce parasite diversity by facilitating competitive exclusion of co-infecting parasite clones, as has been found in the rodent-malaria system (Råberg et al. 2006).

Finally, an understanding of within-host parasite communities is particularly important for interpreting the costs of resistance that form the basis for many EI studies. Koskella et al. (2012) found that bacterial hosts exposed to multiple-phage environments paid higher resistance costs than those exposed to single-phage environments, presumably due to the accumulation of multiple costly resistance mutations, each of which was associated with resistance to a specific phage. Therefore, EI studies must use caution when interpreting results of immune assays that might reflect differences in the diversity of parasite communities challenging hosts.

4.3.4 Parasite Ecology/Evolution

Studies in EI have almost exclusively focused on the importance of immunological variation to the host individual or species (i.e. the organism with the immune system of interest). A glaring gap in EI is a lack of attention paid to the fitness of the parasite in that host. Many properties of the host, including immunological variation, are vital to the parasite's ability to establish and obtain resources needed for development and reproduction (Schmid-Hempel 2011). The ecological factors that shape immunological variation in animals also shape the conditions that select for traits of the parasite, or produce the ecological niches that the parasites occupy (Poulin 2007). Thus, research in EI can contribute tremendous insights to parasite ecology and evolution, as well as our understanding of disease ecology. To our knowledge, there have not been any formal attempts to link questions in EI to questions in parasitology. Below, we offer two speculative ideas for how these fields may intersect. Given the multidisciplinary natures of EI and parasitology, we fully expect that many more intersections exist.

Sexual Selection and Parasite Adaptation The Hamilton-Zuk Hypothesis proposed that secondary sexual traits (e.g. ornaments involved in mate choice) could serve

as indicators of parasite resistance, which would provide signals in courtship that are connected to a fitness advantage (Hamilton and Zuk 1982). In other words, this hypothesis describes a way for secondary sexual traits to provide "honest signals" of the health status of a potential mate. This hypothesis has been of interest to EI, because immune function is a logical pathway for linking parasite resistance with key life-history traits (Owen et al. 2010). For example, Baeta et al. (2008) demonstrated that experimental supplementation of carotenoids in the diet of blackbirds (*Turdus merula*) improved male bill coloration (a secondary sexual trait) and resistance to an intestinal parasite (*Isospora*). In contrast, parasitized males without the supplemented carotenoids had dull beak color and higher parasite loads. Thus, diet quality (an ecological variable), parasite resistance and a sexually-selected indicator trait all appeared to interact.

Parasitological interest in the Hamilton-Zuk Hypothesis has mostly consisted of how to measure/interpret parasite burden and distribution, relative to the host's secondary sexual traits (John 1997; Garamszegi and Moller 2012). An unexplored link to parasitology is the potential for indicator traits to influence parasite adaptation and evolution. If parasite resistance is coupled to "honest signals" in sexual selection, then hosts will hypothetically be able to avoid infected individuals and choose effective defensive genes. These host traits would reduce parasite transmission and the ability of the parasite to establish (i.e. produce a fitness cost for the parasite). An expected outcome might be selection for parasite traits that do not trigger these signals in the host. Thus, we might ask: do indicator traits select for decreased virulence, so the parasite is not "revealed" by the sexual trait? Alternatively, do indicator traits select for parasites that can decouple from the "honest signal" in the host, or select parasite traits that allow the parasite to manipulate the host signal?

Resource Trade-Offs and Parasite Host Switching Perhaps the most frequently explored concept in EI is that immune function is energetically expensive and limited by physiological resources that create necessary trade-offs between immune function and other life history traits. This concept is explicitly focused on the condition and fitness of the host organism. However, if we consider that the host represents an immunologically-defended resource for the parasite, we might ask how these host trade-offs could provide opportunities and/or selection pressures for the parasite? In other words, if immunological defense can be compromised due to internal partitioning of limited resources, then perhaps ecological events that promote compromised immune function also create opportunities for parasites to reproduce, spread or switch to new host species. For example, during periods of nutritional stress (e.g., drought), do host populations experience a general decline in immune defense that allows parasites to spread, or switch to new host species that were previously resistant to invasion? Furthermore, does host heterogeneity in immunity, as caused by nutritional stress, influence the evolution of parasite virulence (Williams 2012)?

4.4 Challenges for the Future

Ecoimmunology studies that are relevant to host-parasite interactions will ideally start with a basic understanding of the nature of immune involvement in the particular host-parasite interaction of interest. However, one of the greatest challenges with this starting point is the currently limited knowledge of the immune system in non-model organisms, and the role of the immune system for a particular host-parasite interaction. Emerging and increasingly cost-effective genomic techniques are providing one powerful approach for characterizing host-parasite-immune interactions in non-model organisms (Pedersen and Babayan 2011). However, the inability to generate immune reagents (antibodies, etc.) that effectively work across species and in non-model organisms will continue to limit progress in this field. Furthermore, the complexity of immune responses, and their varied role in protection, for well-characterized diseases in animal models (e.g. mice) underscores the difficulty in simplifying the immune system to a few key measures. Even for well-controlled, inbred laboratory systems, which likely have significantly less variation than in free-living animals, it has proven very difficult to identify discrete immunological pathways that are vital to host fitness.

How should we proceed in the face of these limitations? Graham et al. (2011) provide a useful framework for designing ecoimmunology experiments in the context of parasitology. In particular, the authors recommend combining three key components in order to evaluate the benefits and costs of immune phenotypes in the context of a host-parasite interaction: (i) a measure of immune response, or phenotype, (ii) parasite density, and (iii) host fitness.

The immune responses characterized in EI studies do not necessarily need to be involved in the particular host-parasite interaction under study. However, any characterization of a generalized immune measure must correlate with specific immune responses to the parasite of interest. For example, in their study on Soay sheep, Graham et al. (2010) found that total immunoglobulin G, antibodies to ribonucleoprotein, and antibodies to the parasitic nematode *Teladorsagia circumcincta* were all positively correlated with individual antibody responses to nuclear and cytoplasmic antigens (antinuclear antibodies, or ANAs). Therefore, the authors conclude that ANA antibodies, which they link with two components of fitness, may broadly reflect general antibody responsiveness.

To assess parasite fitness, relevant parasite taxa must be identified, standardized methods should be used to determine parasite density, and baseline data should be gathered that characterize the typical population dynamics of the parasite. These measures are necessary to enable researchers to determine if suspected ecoimmunological interactions in the host species have an effect on parasite fitness, or host defense. Fortunately, many resources exist for finding/measuring diverse parasites (Roberts and Janovy 2000). An important challenge for future studies in EI will be to connect broad, cross-taxonomic immune measures with specific host-parasite interactions.

Host fitness is notoriously difficult to quantify for free-ranging animals, but the measurement of fitness proxies, such as annual reproductive success, is a step in

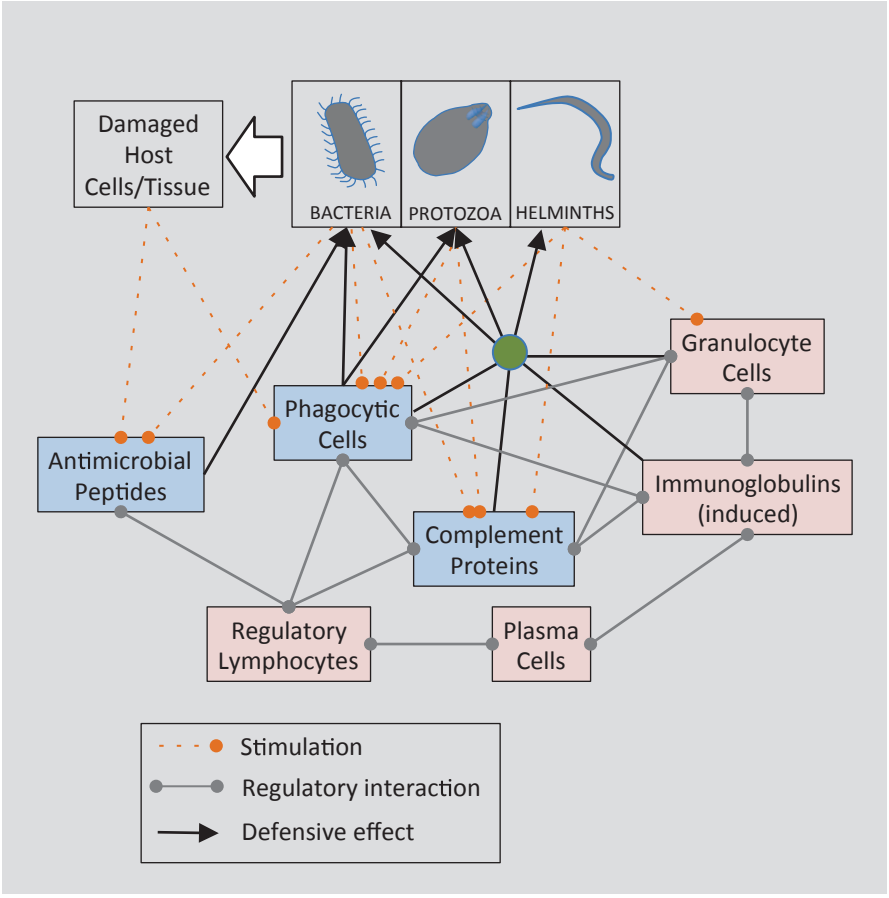
the right direction (Graham et al. 2010). Overall, the inclusion of fitness measures, as recommended by Graham et al. (2011), will help to determine whether the measured immune variable is meaningful to a host-parasite interaction: if hosts that invest highly in immune function have fitness advantages in the presence, but not absence, of the parasite of interest, then individual investment in that immune response is at least correlated with a component of protection.

Regardless of the methodological hurdles, we feel that significant progress can be made in EI simply by broadening the host-centric perspective to include effects of host immune responses on parasites. This shift in perspective takes an important step toward equal consideration of both players in the host-parasite interaction, and has the potential to transform both the fields of EI and parasitology. We hope that ecoimmunologists will begin to see parasites as an exciting way to provide an ecological/evolutionary context for studies of immune function, rather than as a complication to be avoided in pursuit of broader questions. Similarly, we hope that parasitologists will recognize how EI can be uniquely informative for ongoing questions about parasite ecology and evolution. Indeed, we believe that ecological immunology and parasitology are two fields of research that, joined, are poised to produce remarkable insights for biology.

BOX 1—Glossary of Terms

Adaptive immunity—	Cells and proteins that are actively regulated to adjust the recognition of parasite molecular patterns, focus defenses on specific parasites and maintain immunological "memory".
Antigen-presenting cells—	Phagocytic cells (e.g. macrophage, dendritic cell and B-lymphocyte) that process and present parasite-associated molecules to helper T-lymphocytes via the MCH class II molecule. This is a key first step in the adaptive immune response.
Antimicrobial peptides—	Innate immunity proteins that can directly kill microbial parasites (viruses, bacteria, and fungi).
Cellular effector—	Cells involved in the immune response (e.g. phagocytic cells, lymphocytes, granulocytic cells).
Complement system—	Multiple innate, precursor proteins in circulation that engage one another in a cascade that results in a protein complex that can lyse target cells. Products of the cascade also mark parasites and attract cellular effectors (e.g. macrophages and neutrophils) that attack the parasite.

Cytokines—	Cell signaling molecules that attract cellular effectors and modulate target cell activity.
Granulocytes—	Phagocytic cells that produce and release toxic, or proteolytic compounds that damage parasite cells/tissues (e.g. mast cell, neutrophil, basophil, eosinophil).
Humoral effector—	Soluble molecules involved in the immune response (e.g. antimicrobial peptides, complement, immunoglobulins).
Immunoglobulin (Ig)—	(= antibody) Proteins produced by B-lymphocytes as part of the adaptive immune response. These proteins have regions that are highly variable in structure and can bind to parasite-associated molecules. The Ig molecules can neutralize the function of parasite-associated molecules and mark parasites for attack by complement, phagocytes and granulocytes.
Innate immunity—	Cells and proteins present prior to infection that are unable to adjust to molecular patterns of parasites, but have some genetically determined parasite recognition.
Lymphocytes—	Innate (e.g. natural killer cell) and adaptive (T- and B-lymphocytes) immunological cells. T-lymphocytes are involved in regulation of the immune response. B-lymphocytes are responsible for production of immunoglobulin.
Lymphoid tissues—	Tissues involved with maturation of lymphocytes and bringing parasite-associated molecules into contact with lymphocytes to initiate an adaptive immune response.
Parasite-associated molecular patterns—	Conserved molecules found on parasites/pathogens that may be recognized by innate immune effectors (e.g. lipopolysaccharide molecules on gram-negative bacteria that are recognized by toll-like receptors of macrophages).



BOX 2—Overview of Immune Defenses Against Parasites

Here we diagram some of the interactions known to occur among parasites and components of the host immune response during the first (primary) exposure. Diverse parasite taxa typically stimulate the host immune response via signals (dashed, orange lines) from (i) damaged host cells/tissues, or (ii) host recognition of parasite-associated molecules (e.g. toll-like receptor binding to bacterial lipopolysaccharide). These signals affect the activity of phagocytic cells (e.g. macrophages) and defensive peptides (e.g. complement) that are often considered elements of the innate immune system (blue boxes). In turn, those innate effectors signal (grey lines) an adaptive immune response (pink boxes) via cytokines that result in the production of regulatory cells (e.g. helper T lymphocytes) and defensive peptides (e.g. immunoglobulin) that

specifically target the parasite. Both the innate and adaptive immune effectors have defensive effects on parasites (black arrows) (e.g. lysis). Importantly, the activities of those various effectors (i) occur simultaneously, (ii) are regulated through continual feedback (grey lines), and (iii) can act synergistically (green circle).

For example, bacteria may stimulate phagocytosis via activation of toll-like receptors on phagocytic cells (e.g. macrophages). Those phagocytic cells activate helper T lymphocytes through antigen presentation via the MHC class II molecule, which would in turn coordinate the production of immunoglobulins specific to the bacteria. Simultaneously, the complement protein cascade could be initiated against the bacteria, causing direct damage to the parasite and also producing cytokines that would attract more immunological cells to the site of infection. Finally, the immunoglobulins produced by B lymphocytes (plasma cells) would bind to remaining bacteria. Those bacteria-bound immunoglobulins could directly affect the bacteria (e.g. neutralizing key surface receptors), promote destruction of the parasite by signaling phagocytosis (e.g. Fc receptors on macrophages), or trigger more complement activation (Classical pathway).

The key messages illustrated are that (i) diverse parasite taxa can trigger the same pathways in the immune response, or unique pathways, (ii) multiple components of the immune system from both the innate and acquired "arms" are simultaneously involved at all stages of an immune response and (iii) each element of the immune system is engaged in active communication and co-regulation with other elements.

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Chapter 5
Immune-Neuroendocrine Integration and Its Evolution

D. Malagoli and E. Ottaviani

Abstract In the present chapter, the immune-neuroendocrine integration and its evolution will be analyzed. The attention will be especially focused on the evolution of immunological memory in vertebrates and how this have modified the relationships between neuroendocrine and immune systems. We will analyze whether indications provided by eco-immunologists can be of help in describing the evolution of the immune-neuroendocrine system, and verify whether such indications can be applied to all metazoans despite the diversity between vertebrate and invertebrate immune functions and mediators.

Keywords Evolution · Eco-immunology · Stress · Immunity

5.1 Introduction

Among other basic assumptions, eco-immunologists consider that in the absence of immune challenge, the energy expenditure for immune responses must be minimized. The allocation of different amounts of energy on the basis of functional status may be achieved by mean of trade-offs, that is the transfer of energy availability between different systems and components (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000). This fundamental characteristic has been considered as the basis of the functioning of mammalian immune system, which, however, represents a very particular model of the immune system and relies heavily on lymphocyte activity and adaptive immunity. Despite its peculiarity, mammalian immunity is deeply integrated with neuroendocrine functions, as it has been proposed for all metazoans (Ottaviani et al. 2008).

In the present chapter, we will discuss immune-neuroendocrine integration and its evolution, focusing our attention especially on the evolutionary jump of immunological memory in vertebrates. We will analyze whether indications

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3_5,

provided by eco-immunologists can be of help in describing the evolution of the immune-neuroendocrine system, and verify whether such indications can be applied despite the diversity between vertebrates and invertebrates.

5.2 Interactions Between the Immune and Neuroendocrine Systems

Several papers report the existence of a bidirectional regulatory circuit between the immune and neuroendocrine systems, in which the systems communicating with each other produce and respond to similar molecules, in order to cope with potentially damaging internal and external agents (see for reviews Weigent and Blalock 1987; Blalock 1989; Ottaviani et al. 1997b; Demas et al. 2011). The immune cells are able to produce neuroendocrine hormones, which when they bind to specific receptors on immune cells modulate their activity. At the same time, products of the immune system act on cells of the neuroendocrine system modifying their functions (Weigent and Blalock 1994).

Today, the cross-talk between immune and neuroendocrine systems has become well-known as a result of pioneer research undertaken in various laboratories. Blalock and Smith (1980) showed that during the production of interferon-γ, human leukocytes co-ordinately express a peptide antigenically related to adrenocorticotropin hormone (ACTH) and present the structural and biological characteristics of the pituitary-derived ACTH. Berkenbosch et al. (1987) has shown that interleukin (IL)-1 can activate the hypothalamic-pituitary-adrenal (HPA) axis and promote the release of corticotropin-releasing hormone (CRH) from the hypothalamus. Such observations show that the term "stressor" may include all those conditions which result in an increase of the production and release of IL-1, *e.g.* the exposure to bacteria, viruses, or antigens that ultimately result in the stimulation of the immune system. Moreover, the effects of cytokines on the HPA axis have been confirmed and extended. Other cytokines, such as IL-2, IL-6 and Tumor Necrosis Factor (TNF)-α have also been shown to be able to increase plasma ACTH levels and to function as neuromodulators (Naitoh et al. 1988; Sharp et al. 1989; Karanth and McCann 1991; Schöbitz et al. 1994). Finally, Ader et al. (1990) suggested that the immune system is regulated by the central nervous system either directly or by the neuroendocrine axis, *i.e.* the hypothalamic-hypophysial system or the autonomic nervous system.

This theory has been the fundamental framework for comparative immunologists and neuroendocrinologists, whose first aim has been the search for a comparable overlap in vertebrates other than humans or in simpler organisms. Indeed, mediators appear to be shared by immune and neuroendocrine responses in non-mammalian vertebrates and also in invertebrates. Vertebrate leukocytes and invertebrate immunocytes both produce biologically active pro-opiomelanocortin (POMC)-derived peptides, such as ACTH (Blalock 1989; Ottaviani et al. 1992, 1997b). ACTH-like molecules have been found in different tissues and immunocytes of invertebrate models, especially in molluscs (Ottaviani et al. 1999; Stefano et al. 2002). With

regards non-mammalian vertebrate species, the presence of ACTH-like molecules has been detected using immunocytochemical and cytofluorimetric studies in the macrophages of bony fish, urodelan and anuran amphibians, reptiles and birds, while in lymphocytes ACTH-immunoreactive material has been observed only in tetrapods (Ottaviani et al. 1992). The presence of mRNA encoding for ACTH-like material in vertebrate immune-related cells has been confirmed by *in situ* hybridization (Ottaviani et al. 1995a), Northern blot and RT-PCR (Murao et al. 1998). Comparable results have been collected for a variety of molecules shared by the neuroendocrine and immune systems, such as those involved in stress response, *i.e.* CRH, glucocorticoids and biogenic amines.

Selye (1950) defined stress as any deviation from homeostasis, stating that "any change affecting a living organism is a stressor, as it elicits some efforts to resist and, hence, at least mild manifestations of an alarm reaction". According to Selye's definition, every organism must be able to counteract countless stressors continuously in order to survive. Moreover, since the concept of homeostasis is an implicit condition for survival, not surprisingly the stress response appears to be based on ancient and conserved mechanisms and mediators. In some cases, the stress response in invertebrate and vertebrate models is mediated by the same molecules in a similar order and pattern (Ottaviani and Franceschi 1996). In agreement with the tinkering model elaborated by Jacob (1977), we have proposed that evolution refined the immune-neuroendocrine functions by introducing hierarchical levels of organization in the stress response (Malagoli et al. 2004). According to the model, the basic components and mediators have been conserved and incorporated into new circuitries and axes (Ottaviani et al. 1998b). Three levels of stress response organization are seen (Malagoli et al. 2004), reflecting the increasing complexity observed during metazoan diversification:

i. the first level is observable within cells, exemplified by the molluscan circulating immunocytes or the vertebrate immune-neuroendocrine cells, such as lymphocytes and macrophages (Blalock 1984; Ottaviani et al. 1993);
ii. the second level is seen in organs, *i.e.* glands such as thymus or pancreas that may respond locally to a stressor;
iii. the third level is "organismic"; in vertebrates, this is the HPA axis (constituted by hypothalamus, pituitary and adrenal glands) that ensures coordination among other organs and systems.

The first level of stress response can be analyzed in invertebrate models and in vertebrate lymphocytes (Ottaviani et al. 1992). Studies performed in molluscs have shown that mammalian CRH and ACTH provoke the release of biogenic amines by molluscan immunocytes (Ottaviani et al. 1998a), mimicking at the molecular level the HPA stress response axis, which CRH-like, ACTH-like and biogenic amines seem to reproduce. From these findings, a simplified scenario emerges that involves the immunocyte alone harbouring all the molecules that in vertebrates are also distributed in separate organs not found in molluscs. In these respects, by exerting immune-surveillance and harbouring stress-related molecules, the molluscan immunocytes can be depicted as a multifunctional cell as well as an example of an immune-neuroendocrine player (Ottaviani 2011).

However, immune-neuroendocrine integration in immunocytes is not only represented by the co-existence within the same cell of immune functions and stress-related mediators. As for vertebrates, immune-related molecules are also involved in the stress response in molluscs, confirming that the cross-talk between the immune and neuroendocrine systems is equally present in invertebrates. Experiments performed in molluscs have demonstrated that human IL-2 and CRH alone provoke a significant release of biogenic amines from immunocytes. Moreover, the pre-incubation of the hemolymph with human IL-2 significantly reduces the release of molluscan biogenic amines that follows a further stimulation with human CRH (Ottaviani et al. 1994). Analogous observations have been registered for human IL-1α, IL-1β, TNF-α and TNF-β, suggesting that the competition among cytokines and CRH is probably due to the promiscuity of a receptor able to bind more than one ligand (Ottaviani et al. 1995b). Beside cytokines, also human growth factors such as platelet-derived growth factor (PDGF)-AB and transforming growth factor (TGF)-β can stimulate the stress response in molluscan immunocytes. Indeed, the biogenic amine release induced by growth factors is mediated by the CRH-ACTH-biogenic amine axis (Ottaviani et al. 1997a, 1998a).

The second and the third level of organization in the stress response have been hypothesized on the basis of observations in fish. Experiments performed in the channel catfish *Ameurus nebulosus* (Malagoli et al. 2004) have shown the presence of a *proCRH* gene showing four exons and three introns for a total length of 1416 bp. ProCRH immunoreactive molecules were detected in the central nervous system, head kidney and exocrine pancreas. Using an immune-related stressor like lipopolysaccharide (LPS) for 15 and 120 min., an increase in proCRH immunoreactivity in brain sections was detected after 15 min, but not after 120 min. Conversely, the increase in the peripheral glands persisted for the entire period of the treatment. These findings support the assumption that stress response is hierarchy- and time-regulated. This behaviour is in agreement with our and other data reported in literature in different animal models (Putti et al. 1999; Varsamos et al. 2003) and it may be hypothesized that it respects the model of energy trade-offs between different systems. Experiments on the catfish would suggest that the central nervous system is involved in the first phase of the response. However, after a few hours, only elements of the endocrine system still appear activated. This way of organizing a stress response could be a means of limiting the involvement of the nervous system and saving energy directed towards a persistent stressor, at least in the short term (Malagoli et al. 2004).

5.3 The Evolution of Immune and Neuroendocrine Interactions Under the Lens of Eco-immunology

The cross-talk between immune and neuroendocrine system has been a fundamental advantage as it is conserved during the diversification of such different organisms as snails and humans. One possible explanation could be that the cross-talk *per se* is the hub on which the diversification of nervous, endocrine and immune

systems is hinged. This supposition mainly derives from experiments performed in vertebrates or in molluscan *taxa*, but the partial overlap between immune and neural mediators can also be seen in other groups. For example, an overlap of immune and neural functions has been reported in the insect *Drosophila melanogaster*, where the cytokine Unpaired (Upd) and the related JAK-STAT pathway seem connected to long-lasting memorization, but not in less stable forms of memory (Copf et al. 2011). The observation is significant, because although the cytokine repertoire of the fruit fly *D. melanogaster* is significantly smaller than that of mammals, and the fruit fly belongs to a highly diversified invertebrate group that is evolutionarily distant from molluscs, there is still an overlap between the immune and neural functions, strengthening the hypothesis that the cross-talk between said systems is the root rather then the result of diversification.

Various fundamental concepts introduced into eco-immunology, such as trade-offs, modulation of the immune response on the basis of energy availability, and immune aging, have been developed on the basis of observations performed in vertebrates. However, the basic principles of eco-immunology have been proven to be of general validity and applicable to the majority of multi-cellular organisms. If we accept that the immune and neuroendocrine systems present a basic and unbreakable connection, then the trade-off between these components should be particularly evident. Indeed, it is commonly observed that immune efficiency depends on nutritional status (Martin et al. 2008), but also that an artificially increased immune reactivity is detrimental for reproduction (a function largely dependent on endocrine regulation). Experiments performed in *D. melanogaster* have revealed that flies selected for a major responsiveness towards the bacterium *Pseudomonas aeruginosa* are more effective in removing the pathogen, but display a reduced egg viability and longevity. Moreover, when the stressor is removed, the new populations rapidly lost the major responsiveness in favour of a restored egg viability (Ye et al. 2009). As in mammals (Lochmiller and Deerenberg 2000), this observation justifies the view that also in invertebrates the evolution of a highly effective immune system is limited by the costs of maintaining the defence apparatus in the absence of an actual treat (Kraaijeveld and Godfray 1997), given that energy must be shared among different systems. Accordingly, flies selected for a better response against wasp parasitoids were worst competitors for food resources in comparison to wild-type flies (Kraaijeveld and Godfray 1997).

Data and examples reported so far highlight the important connection between the immune and neuroendocrine systems in diverse metazoans, and highlight how eco-immunological conceptualization may help in deciphering this interaction. Apart from among vertebrates, the metazoan immune systems appear to have evolved in the absence of adaptive/acquired immunity. Immune-neuroendocrine integration predates vertebrate diversification and the acquisition of an adaptive component in the immune system. It is therefore important to analyze whether the ancient link between immune and neuroendocrine systems has also been maintained in present-day vertebrates following the appearance of lymphocytes and immunological memory.

5.4 The Evolution of Immunological Memory Within the Frame of Immune-Neuroendocrine Integration

The question regarding the existence of specificity and memory in the invertebrate immune system is still open. Somatic recombination in invertebrates has been observed in evolutionary distant *taxa* such as insects (Watson et al. 2005), molluscs (Zhang and Loker 2004) and the basal deuterostomian echinoderms (Dheilly et al. 2011; Smith 2012; Majeske et al. 2013). There are numerous studies on possible "vaccination" and specificity in pathogen recognition (Roth et al. 2009; Pope et al. 2011), but no definitive proofs of the existence of a widespread immunological specificity and memory have been provided for either protostomian or deuterostomian invertebrates (Hauton and Smith 2007). Vertebrate evolution has been characterized by two whole round genome duplications, and maybe a third took place in teleosts (Workenhe et al. 2010). These fundamental events appear to be crucial for the evolution of lymphocytes and immunological memory (Cooper and Alder 2006). In the basal and jawless vertebrate lamprey, the immune system independently evolved an organization that recalls the T-B lymphocyte network of other vertebrates. Even though in the lamprey there are no T and B lymphocytes, and lymphoid organs such as the thymus are not found, these vertebrates nonetheless present cells with different receptors that appear to cooperate in determining a specific immune response (Cooper and Herrin 2010). On the other hand, the vertebrate immune system appears to be hinged on the function of lymphocytes and the production of antibodies, meaning that we can currently conclude that the co-existence of specificity and memory is a feature of vertebrate immune system. The passage from an innate to a memory-based immune system must have respected all the constraints that we have analyzed so far in terms of trade-off and energy requirement. However, while there is solid evidence to consider the evolution of the interconnection between immune and neuroendocrine mediators in the innate immune system, the lack of analogous systems outside vertebrates does not allow evolutionary considerations. It is worth noting that immunological memory is a feature found in all vertebrates, but the role of memory in immunity does not appear to have the same importance in all vertebrate *taxa*. In teleost fish, for instance, there is an IgM class of immunoglobulins, and the secondary response following the re-exposition to a immune challenge does not follow the same trend as that seen in mammals (Star et al. 2011). In these respects, the discrimination between constitutive and conditional costs of immunity should not be forgotten; the first concerns the evolution/development of an immune system, while the latter relates to its actual use (Van Boven and Weissing 2004; Schimd-Hempel 2005).

In general, the secondary immune response in vertebrate *taxa* cannot be described using the model of mammals as a paradigm. In teleosts, the most widespread and diversified vertebrate group which probably evolved after a third whole round genome replication (John et al. 2009; Workenhe et al. 2010), the "mammalian model" today appears hardly applicable. For instance, Toll-like receptor diversification appears more relevant in bony fish (Coscia et al. 2011), while in the Atlantic codfish the absence of MHC-II group and CD4+T lymphocytes has

been observed (Star et al. 2011). This fundamental observation indicates that the vertebrate immune system is a highly evolvable system, and that its various components can be expanded or reduced to construct different, but still functional, immune responses (Star et al. 2011). Secondary responses in chondrocytes, bony fish, amphibians and reptiles appear to be sluggish and less relevant compared to those found in birds and mammals. In the amphibian genus, *Xenopus,* the affinity maturation of immunoglobulin is poorer than in mammals (Robert and Otha 2009), and, as in other poikilotherm vertebrates, the immune functions are strongly influenced by external temperature (Robert and Otha 2009). In reptiles, too, the humoral response after a first exposure to a pathogen is much slower (about 6–8 weeks) than that in mammals and birds (1–2 weeks) (Work et al. 2000; Zimmermann et al. 2010a, b). Moreover in reptiles, humoral responses do not increase in titre or in binding affinity during or after a second challenge (Work et al. 2010). The evolutionary history of vertebrates indicates that reptiles and birds can be grouped into the monophyletic group of sauropsid. Therefore, the similarities observed between birds and mammals have to be considered the consequence of a convergent evolution. This suggests that a highly refined and relevant secondary humoral response may represent an advantage and be positively selected only when specific features are present.

Despite a different phylogenic history, birds and mammals are the only vertebrates presenting Peyer's patches and lymph nodes with germinal centers (Zimmermann et al. 2010b). Moreover they are the only tetrapods that show affinity maturation (Zimmermann et al. 2010b), while their adaptive immunity seems more responsive and massive than that in other tetrapods. The principal characteristic that birds share with mammals but not reptiles (*i.e.* the other sauropsids) is tachimetabolism, which is closely interconnected with homeothermy. The dependence on temperature of the immune response in cold blooded vertebrates has been reported on several occasions (Nelson 2004; Martin et al. 2008). A constant temperature could be considered the starting point for the evolution of an immune system adapted to specific working temperatures. However, temperature fluctuations can also occur in homeotherm vertebrates, and in some cases these are connected with the immune response itself, *e.g.* fever (Zimmermann et al. 2010b). If we consider the concepts of trade-off and energy expenditure, the similarity in the immune response of birds and mammals may be imputed to tachimetabolism itself. In tachimetabolic organisms, the global amount of energy expenditure is higher than in cold-blooded vertebrates (Lochmiller and Deerenberg 2000), and this could indicate major trade-offs between the various systems. As mentioned above and further discussed in Chap. 6 of the present book, immunological memory may be a costly feature to set up, but subsequently it can then be utilized only when needed. In these respects, it is worth mentioning that basal metabolic rate of adult lymphocyte-deficient mice is higher than that of wild type mice (Råberg et al. 2002) suggesting that metabolic advantage could have driven the evolution of an immune system combining the innate and the acquired component (Råberg et al. 2002). In specific situations, the amount of energy that can be allocated to immune response in tachimetabolic animals is relevant. An important secondary response acts in concert with innate immunity in order to rapidly and specifically contrast a pathogen. In this view, humoral adaptive immunity could be seen as an accessory to the innate, cell-mediated immunity. This accessorial feature would

have significantly increased its importance upon acquisition of tachimetabolism, energizing an already existing apparatus. The convergent evolution of a powerful immune component in birds and mammals is not exceptional in the evolution of vertebrate immunity. For instance, all vertebrates possess mast-cells, but only amniotes and teleosts independently evolved histamine-endowed mastocytes with a role in regulating inflammatory response (Mulero et al. 2007).

The adaptive branch of the vertebrate immune system is mingled with and dependent on innate immunity in its functions (Beutler 2004). The innate immune components appear less diversified than the adaptive elements (Workhene et al. 2010). The description of adaptive immunity as a variedly diversified extension of innate immunity is in agreement with this observation. Innate immunity relies on different cell types in diverse animals, but it is always referable to the activity of a circulating cell with phagocytic activity that can produce anti-pathogen mediators. Acquired immunity, on the other hand, relies on a vertebrate-specific cell type, *i.e.* the vertebrate lymphocytes or the lamprey counterparts (Cooper and Alder 2006).

Blalock (1984) defined the mammalian lymphocyte as an immune-mobile brain, highlighting the significant overlap between neuroendocrine and immune functions also in organisms that have a pivotal immune component in adaptive immunity. Several examples of reciprocal influence between immune and neuroendocrine systems can be found in vertebrates. Fever is a typical manifestation of an inflammatory process that is activated by circulating cytokines operating on the hypothalamus (Conti et al. 2004). Fever is observed in tachimetabolic and poekilotherms (Zimmermann 2010b), with the latter exhibiting behavioural activation to increase body temperature (Kluger et al. 1975; Merchant et al. 2007). It has been proposed that the hypothalamus—the principal component bridging neural and endocrine functions—is also a fundamental regulator of immune response (Berczi et al. 2009). The same authors have also suggested that the HPA axis exerts opposite effects on innate and acquired immunity, enhancing the former while suppressing the latter. On the other hand, the immune system can influence neuroendocrine functions. In mammals, LPS injection can induce a depressive-like behaviour (Frenois et al. 2007; Danzer et al. 2008). Seeking to increase knowledge on the transition from sickness to symptoms of depression following an immune challenge, Bay-Richter et al. (2011) have found that after LPS injection, the inflammatory cytokines IL-1β and IL-6 display a different trend in serum and cerebrospinal fluid. Also in rat brain, different amounts of the two cytokines are produced 2 and 24 h after injection, so justifying the observation that an immune challenge can result in two behavioural states, a transitory sickness and a long-lasting depression (Bay-Richter et al. 2011). This result resembles the observation reported above in channel catifish *A. nebulosus*, where the peripheral and central expression of CRH are diversely influenced at different times of exposure to LPS (Malagoli et al. 2004). These examples among the many available confirm that immune, nervous and endocrine responses are constantly intersecting. Overall, when speaking of immune, nervous and endocrine functions, we are probably considering just one aspect of a single system that has become highly diversified among metazoans and in a special way among vertebrates.

5.5 Conclusions

Immune, nervous and endocrine systems can be easily represented as separated aspects of a single and more comprehensive system. Rather than speaking of individual components integrated into the immune-neuroendocrine system, we suggest considering the elements as specialized aspects of a single system. We speculate that complex immune-neuroendocrine responses have evolved from functional units resembling some of the present-day molluscan immunocytes. Intriguingly, a cell that appears to be exclusive to vertebrates, the lymphocyte, also presents some characteristics of an immune-neuroendocrine cell. It seems that immune-neuroendocrine integration is the common denominator, rather then the sum, of the diversification of the immune, neural and endocrine components. The intrinsic capability to optimize energy costs, which may be related to pleiotropy and functional redundancy, has made the immune-neuroendocrine system into an evolvable unit that has diversified greatly in different animal taxa, while maintaining intact the cross-talk and trade-offs among its various components.

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Chapter 6
Thymic Maturation and Programmed Cell Death

Daniela Quaglino, Alice Accorsi, Federica Boraldi and Enzo Ottaviani

Abstract The thymus plays a crucial role in the development and maintenance of the immune system, being the main site of T cell differentiation and maturation throughout life. Associated to dramatic structural changes, its function seems to markedly diminish with time, never the less, there are several data indicating that, despite organ atrophy, at least part of the thymus remains active throughout one's lifetime. In the last decades, several studies, aiming to understand the significance of age-dependent changes in thymic structure and function, highlighted the concept that developmental and maturational stages strongly depend on the balanced and coordinated occurrence of life and death options. In particular, programmed cell death represents a fundamental requirement in order to assure a proper functionality of the immune response and to avoid the formation of uncontrolled and potentially self-damaging lymphocytic clones. By contrast, the time-dependent thymic atrophy is due to progressive replacing of lymphoid with adipose tissue. In the light of the increased knowledge on the factors/mechanisms controlling the process of adipogenesis, it could be suggested that fat accumulation in the thymic stroma might not be considered a passive, deleterious consequence of aging, but instead a potential source of molecules with various biological functions. Therefore, thymus represents a very interesting model in terms of energy expenditure and trade off, tissue homeostasis, immune defence and disease escape. The implications of changes in thymic structure, in the ratio of proliferation and programmed cell death as well as the occurrence of fat involution still represent an open question and will be discussed in the present chapter.

Keywords Thymus · Apoptosis · Involution · Immune system · Evolution

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3_6,

6.1 Vertebrate Thymic Structure: From Embryogenesis to Complete Growth

The thymus is present in all vertebrates, although it markedly differs among species in terms of number per animal, anatomical position, structure of lobes, developmental origin and maturation. Most of the studies have been performed in mammals as mice and humans, where the organ is anatomically located in the upper anterior portion of the thorax, just behind the sternum and in front of great vessels, whereas in teleosts (the most widespread and diversified group of bony fish) the thymus shape is not homogeneous (Zapata et al. 2006) and an early primordium is usually observed lining the pharyngeal cavity.

During embryogenesis, the human thymus arises bilaterally from the third and fourth branchial pouches and contains elements derived from all three germinal layers. Its development begins in the 6th gestational week (gw), but up to the 9th gw it remains comprised exclusively of epithelial cells. It is only after the 10th gw that lymphoid cells migrate from foetal liver and bone marrow starting the colonization process. After separation of the gland into lobules, the parenchyma progressively differentiates into two distinct areas: the cortex and the medulla. From the 15th gw up to birth, the thymus grows rapidly and reaches its greatest weight (approximately 15 g) in relation to body mass. After attaining its maximum size (about 25 cm^3), at the end of the first year, the thymus undergoes a time-dependent involution that is responsible for the progressive reduction of the amount of true thymic lymphoid tissue, without changes in the gross size of the whole organ, because of the parallel expansion of connective tissue and fat (Nishino et al. 2006).

Extensive evidence suggests that an age-associated regression occurs in perhaps all vertebrates that have a thymus, including birds, amphibians and teleosts, indicating that this is an evolutionary ancient and conserved event (Shanley et al. 2009). Never the less, these changes do not seem to occur at the same extent in all species, since in mice, for instance, but not in rats, there is a true loss of thymic tissue with significant reduction in organ dimensions as well as in the number of T cells leaving the thymus, the remaining tissue being also less efficient and producing fewer cells than expected (George and Ritter 1996). In primitive sharks it has been observed that the thymus does not undergo involution (Good et al. 1966), whereas in zebrafish the thymic involution is parallel to sexual maturation, but it does not slow down the lymphopoietic activity (Zapata et al. 2006). In the frog *Xenopus laevis*, the thymus undergoes a first involutive process before the metamorphosis of the tadpole, when up to 90% of lymphocytes leave the thymus (DuPasquier et al. 1989). This event is considered fundamental for avoiding auto-immunity at the adult stage (Rollins-Smith et al. 1997), and is followed by an additional colonization by stem cells and a second histogenesis (Bechtold et al. 1992). In reptiles, animals that are ectothermic as fish and amphibians, but are amniotes, as birds and mammals, thymic involution is connected with seasonal cycling and not only with aging (Zimmermann et al. 2010). The development of avian thymus reflects that described for mammals, but studies on the domestic chicken, *Gallus domesticus*, indicated that the thymic involution in birds is characterized by the infiltration of connective rather than fat tissue (Franchini and Ottaviani 1999).

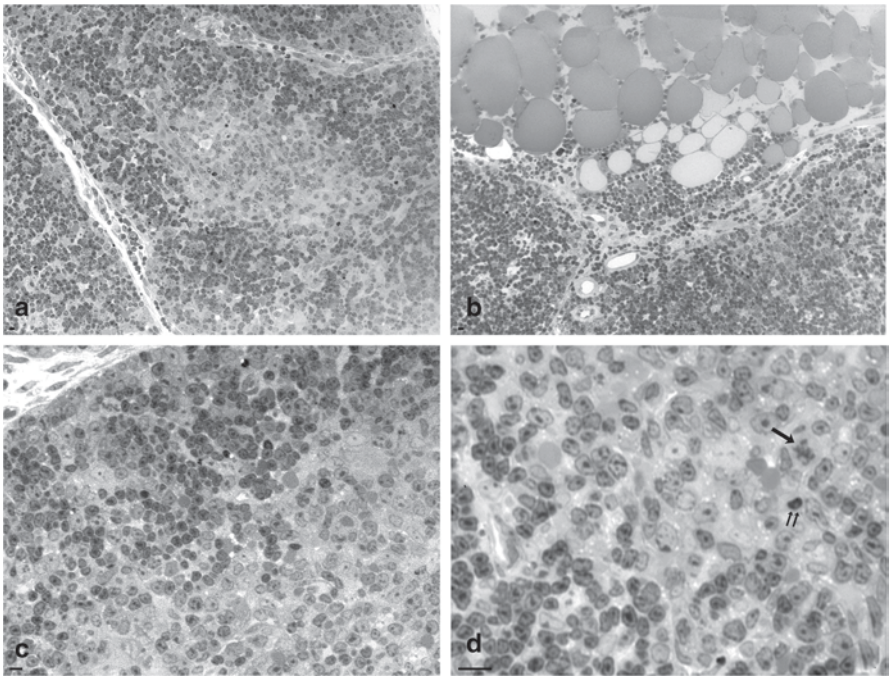

Fig. 6.1 Light microscopy of the thymus in newborn (**a**) and in old (**b**) rats. With age, a progressive increase in the amount of adipose tissue lead to organ involution (**b**). The lymphoid tissue is typically characterized by a darker cortex, where lymphocytes are more abundant, and a lighter medulla (**c**) due to the presence of numerous epithelial cells. Mitoses (*arrow*) and apoptotic cells (*double arrow*) can be frequently observed in the thymic lymphoid tissue (**d**). Bar = 10 μm

Histologically, the cortex of mammalian thymus is composed primarily of lymphocytes with a few epithelial and mesenchymal cells, whereas the medulla is mainly composed of epithelial cells with a low number of lymphocytes (Fig. 6.1). The presence of epithelial cells is believed to be fundamental for the thymic parenchyma, since these so called "nurse cells" are functionally essential for the maturation of T lymphocytes (Penninger et al. 1994). However, beside epithelial cells and lymphocytes, the thymus contains a variety of other types of cells including macrophages, dendritic cells, fibroblasts, plasmacells and, in some cases, also myoid cells (Kendall 1991; Hale 2004).

The composition and the three-dimensional architecture of the thymic microenvironment provide a fundamental source of signalling molecules and favour the formation of cell-to-cell contacts, called immunological synapses. These highly specialized structures are capable to influence developing thymocytes by modulating positive and negative selection as well as cell proliferation, expression of cell-surface molecules, and the gene rearrangements required for establishing a wide repertoire of T cell receptor (TCR) specificities (Ritter and Boyd 1993; Reza and Ritter 1994; Rodriguez-Fernandez et al. 2010).

In humans, the lobular architecture of the organ is frequently lost during the third decade of life and therefore in adults the thymus mainly consists of a mass of adipocytes with only scattered areas of lymphoid tissue (Henry and Anderson 1987). These findings suggest that, within the thymus, lymphocytes represent the cell population most susceptible to time-dependent changes, whereas stromal and epithelial cells are well maintained or may even increase. However, changes in cellular organization, in the ratio among different cell types and in cell-to-cell contacts may have deleterious consequences on thymic homeostasis, including a reduction in the niches available for thymocyte colonization and cell death modulation (Minter and Osborne 2003). All these modifications, known as thymic involution, lead to a progressive reduction of epithelial and T cells. Replacement of the parenchyma by adipose tissue leads to a progressive functional atrophy of the thymus, but this process never seems to reach an end point, since small islands of thymic tissue can be found even in individuals over the age of 80 years (Miller 1967).

6.2 Thymus as a Key Organ of the Immune System

Thymus was firstly described approximately 20 centuries ago by Galenum of Pergamon, who firstly noticed that the organ, at that time considered the site of soul, undergoes extensive changes loosing its consistency after infancy. Since then, two millennia have passed before getting further insights into the functional role of this puzzling organ (Gowans et al. 1962; Fichtelius 1963; Ritter and Crispe 1992; Nishino et al. 2006). In the middle of the twentieth century, the importance of the thymus was still completely neglected and its role in the control of the immune response overlooked (MacLearn et al. 1957; Wolstenholme and O'Connor 1960). Thymus was in fact regarded as an epithelial organ, its atrophy being the proof that, during evolution, it appeared useless and redundant (Miller 1994). This misleading view was overcome by the extension to the thymus of the concept of the "disposable soma" (George and Ritter 1996), which anticipated the considerations about the relevance in balancing the energy costs of the immune response, a concept that has been further formalized by eco-immunologists (Lochmiller and Deerenberg 2000).

One of the first report demonstrating in thymectomized mice the importance of the thymus was published by Miller in 1961 (Miller 1961), although these observations were accepted with scepticism. Thymus is now considered the primary immune tissue devoted to the production and the development of T lymphocytes generated from the interactions of haematopoietic stem cells with thymic stromal cells followed by several maturational stages (Table 6.1). Moreover, within the thymus, cells undergo a series of quality control processes allowing the production of cells with accurate and functional TCR repertoires (Miller 2011). TCRαβ lymphocytes, capable to successfully recognize foreign peptides in association with MHC molecules, are generated by positive selection after interacting with ligands of self-peptides presented by the MHC expressed in the cortex and in the medulla of the thymus. By contrast, T cells, with too strong binding affinity to peptides/MHC

Table 6.1 Typical markers of T cells during their development in the thymus

Maturation stage	Markers	Characteristics
Haemopoietic stem cells (HSC)	$CD117^{+}$, $CD44^{-}$, $CD25^{-}$	HSC that can generate a thymocyte after interactions with thymic stromal cells
Double negative 1 (DN1)	$CD117^{+}$, $CD44^{+}$, $CD25^{-}$	T cell precursors not yet committed but able to develop into other thymus-derived lineages (i.e., dendritic and NK cells)
Double negative 2 (DN2)	$CD117^{+}$, $CD44^{+}$, $CD25^{+}$	Cells in which a developmental program is activated leading to a diverse T cell receptor (TCR) repertoire through the association of distinct α and β TCR chains with regions encoded by variable (V), joining (J), and diversity (D) gene segments
Double negative 3 (DN3)	$CD117^{lo/-}$, $CD44^{-}$, $CD25^{+}$	Cell expressing on the cell surface a functional β TCR chain for the β-selection process
Double negative 4 (DN4)	$CD117^{-}$, $CD44^{-}$, $CD25^{-}$	Cells displaying a fully assembles TCR
Double positive (DP)	$CD4^{+}$, $CD8^{+}$	Cells undergo the TCR selection during a brief period of quiescence. Depending on the extent of type of stimulus cells can undergo positive selection, negative selection or death by neglect

complexes expressed by dendritic cells in the medulla, are deleted by negative selection. Mature T cells can then exit the thymus and migrate to peripheral tissues via lymphatic and blood vessels. All these processes are under the control of cytokines and hormones also produced within the thymic microenvironment (Hernandez et al. 2010). With aging there is a decreased ability of thymic cells to respond to cytokines and interleukins, but exogenous administration of IL-7 and/or IL-12 has been demonstrated to rescue, at least in part, organ involution (Hsu et al. 2005).

Furthermore, the observation that the thymus originates from all the three germinal layers is consistent with the discovery that the immune and the neuroendocrine systems are tightly connected by sharing both ligands and receptors and that intrathymic expression of somatostatin and other neuropeptides can proficiently modulate thymocyte development. This crosstalk has been well proved in several animal species as zebrafish, Xenopus, birds, rodents, swines and humans, confirming that these interactions are evolutionary well conserved (Silva et al. 2006).

Beside its fundamental role in thymocyte activation and differentiation, the thymus produces and releases several hormones such as thymosin, thymopoietin, thymulin and the thymic humoral factor (Miller 1974; Dardenne et al. 1980; Goldstein 2007), suggesting that thymic functions have a broader biological significance in addition to that within the immune system.

Thymic function and energy expenditure is mostly relevant during the foetal and perinatal period, when a consistent thymic export is necessary for establishing the size and the diversity of the naïve T cell pool. Thereafter, the cell output markedly declines resulting in a dramatic age-dependent loss of naïve T cell production (Woodland and Blackman 2006). Changes in the hormonal status and/or the reduction of T cell

progenitors colonizing the thymus from the bone marrow could contribute, but not trigger the age-dependent thymic involution.

In all vertebrates, it is well established that T cells undergo a complex process of maturation and differentiation which lead to the development of a broad TCR repertoire (Brearley et al. 1987; Ge and Zhao 2012). This process seems to be rather unique and thymic specific, since thymectomy during human infancy may have consequences on the naïve T cell compartment with reduced CD4+ and CD8+ peripheral T cells counts (Appay et al. 2010).

6.3 Is Thymus an Evolutionary Conserved Organ?

Defence mechanisms aiming to escape from the deleterious consequences of life-threatening *noxae* are present even in unicellular organisms, but the presence of a more specialized immune response can be better observed in multicellular individuals (Litman et al. 2005). For instance, defence mechanisms based on the activity of macrophages are well conserved from invertebrates to vertebrates. However, the occurrence of properly organized lymphoid organs, and of the thymus in particular, came together with the appearance of the adaptive immunity, as a consequence of the development of complex recognition systems based on the presence of wide T and B cell repertoires, immunoglobulins and MHC molecules, which should provide more efficient mechanisms to counteract exogenous *noxae*. In these circumstances, the development of thymic functions appeared an unavoidable obligation for (i) discriminating between self and non-self, (ii) avoiding an excessive/uncontrolled immune reaction and (iii) allowing the presence of a well defined and selected cell population capable to build up a specific, but at the same time, highly variable response. Recently, gene expression analysis demonstrated, in lamprey larvae, the existence of thymoids. These thymus-like structures are located at the tips of gill filaments, a situation that closely resembles that described in vertebrates, even though in lamprey only T- and B-like lymphocytes are present (Bajoghli et al. 2011). This finding is a strong indication that the ancestor of the extant jawed and jawless vertebrates may have had T- and B-like cells, but also anatomically distinct tissues for their development.

Despite its undeniable role in the controlled development and maturation of functional T cells, thymus highlights the importance of the crosstalk between the immune and the neuroendrocrine systems. Immune-neuroendocrine effectors are already present in invertebrates, where they represent a good integration of innate immunity, stress and inflammation (Ottaviani et al. 1998). These observations seem to indicate that, as observed for the neuroendocrine system (see Chap. 5), the thymus integrates different levels of complexity, each one has specific targets and is modulated by different signalling pathways, most of which have not yet been completely disclosed.

If we consider that thymus, quite early in life, undergoes a progressive atrophy, as if its activity could be pleiotropic or redundant, one still puzzling question is

whether thymic functions can be, at least in part, switched or replaced by other tissues (Rocha et al. 1992; Terszowski et al. 2006; Rocha 2007).

More than 30 years ago, it was proposed that the intestinal epithelium could be equivalent to the thymus epithelium and could sustain lymphocyte development (Fichtelius 1968). However, the hypothesis that thymus and the mucosal associated lymphoid tissue, including the gut lymphoid tissue (GALT), might be evolutionarily related (Hein 1994) came from the more recent observations that, in the GALT of mice as well as of humans, a subpopulation of T cells can be generated and that a type of MHC positive selection of T cells may occur (Matsunaga and Rahman 2001). The hypothesis that the mucosal immunity is more primitive and that the thymus evolved later in order to improve systemic immunity (Mowat and Viney 1997), thus representing a structural and functional sophistication, is based on the observation that the thymic epithelium forms a three-dimensional network, through which developing T cells pass as they mature, whereas the intestinal epithelium has a two-dimensional organization that can reduce the efficiency of lymphocyte development. The primordial thymus could have therefore evolved first within GALT and later, probably due to the lack of sufficient space within the lamina propria, into an independent organ, that tremendously increased cell production when started to have direct connections with blood and lymphatic vessels (Cheroutre and Lamboulez 2008). Consistently, in all gnathostomes, the thymus ontogenically develops as an epithelial mass from the anterior portion of the embryonic gut tube (i.e. the pharyngeal pouches), thereafter it migrates down to the area of the breastbone, where it is colonized by lymphocytes, macrophages and dendritic cells from the bone marrow (Nishino et al. 2006). However, recent observations made in lamprey (Bajoghli et al. 2011) and in several non-mammalian models (Ge and Zhao 2012) indicate that evolution of the thymus has been likely driven by changes at the gene-regulatory level, possibly as a consequences of environmental modifications related to land occupancy. In this perspective, information on basal chordates and other invertebrates might help in elucidating the evolutionary origin of thymus (Ottaviani et al. 1998), as well as the possible co-evolution of T-lymphocytes and thymus. The new inputs and perspectives provided by eco-immunology (see Chap. 1) could further address researches focused on the evolution of acquired immunity and on its energetic costs (Malagoli and Ottaviani 2010).

6.4 When Death Means Life

Although controlled cell death may have evolved for morphogenetic and developmental reasons (Vaux et al. 1994), it has also turned out to be an important defence mechanism and within this context it is found in all living organisms, although targets and goals may be different depending on the species and/or the cell types (Ameisen 2005). Typical examples are represented by the response of bacteria to phages and plasmids or by the hypersensitivity reaction of plants, when cells kill themselves to alert neighbouring cells to the presence of potentially harmful *noxae*.

Similarly, many animal cells, when exposed to viruses, undergo apoptosis in order to limit the production and diffusion of new viral particles and perhaps to signal the immune system (Hedrick et al. 2010). These features might be apparently in contrast with the usual assumption that programmed cell death, and in particular apoptosis, does not cause tissue reactions (i.e, inflammation), since cell debris are neatly removed by macrophages and/or other phagocytes. This discrepancy may reflect two different goal objects reached by the same cell death mechanisms: for developmental purposes and maintenance of tissue homeostasis, when inflammation has to be avoided, and as a defence mechanism, when recruitment of cells of the immune system is advantageous.

In agreement with the importance of these mechanisms for survival purposes, several mediators of cell death, as those causing proteolytic activities or mitochondrial uncoupling, such as the interleukin-1β (IL-lβ)-converting enzyme (ICE) and the cell death abnormal-3 (CED-3), appeared to be well conserved among species (Wang et al. 1994; Yuan et al. 1996). A further example is represented by the cell death gene ted-3, that appeared to be highly conserved from nematodes to humans, and by the inhibitor of apoptosis in nematodes, ted-9, whose human homolog is bcl-2 (Vaux et al. 1992; Hengartner and Horvitz 1994). Consistently, human bcl-2 is able to function in cells from worms, insects and mammals by blocking the cell death machinery. However, in the course of evolution, the increased complexity of living organisms led to the development of different forms of cell death, which can be clearly differentiated on the basis of molecular and structural features (Hedrick et al. 2010).

In mammals, for instance, we can recognize at least three types of cell death: type I apoptosis, type II autophagy and type III necrosis (Clarke 1990; Clarke and Clarke 1996; Lockshin and Zakeri 2001), although many other variants (Melino et al. 2005) have been proposed, based on the signalling mechanisms and the execution pathways, thus leading to a functional and molecular classification instead of a morphology-based nomenclature (Galluzzi et al. 2012).

Apoptosis is typically characterized by chromatolysis as a results of caspase activation due to mitochondrial outer membrane permeability, allowing the release of death effectors (intrinsic apoptosis), or to the involvement of death receptors as Fas and TNFR (extrinsic apoptosis) (Green 2003).

Autophagy, as a mean of cell death, is still under debate (Malagoli et al. 2009; Amelio et al. 2011), but morphologically it has been clearly characterized by the presence of abundant double-membrane vacuoles. It is frequently observed in conditions of nutrient and growth factor deprivation or in the presence of abnormal protein aggregates, being considered a way normally used by cells to avoid or at least delay other forms of cell death (Yorimitsu and Klionsky 2005).

Interestingly, apoptosis and autophagy seem to share similar molecular pathways, e.g., Bcl2, FADD, Flip and Atg3 (Thorburn et al. 2005).

Finally, during necrotic cell death, cytoplasmic constituents as endoplasmic reticulum, Golgi apparatus as well as nuclear and plasma membranes are mainly affected. Surprisingly, with the exception of cyclophilin D, whose role has been recently proposed in some experimental models (Baines et al. 2005; Nakagawa et al. 2005), no other clear molecular or biochemical signatures have been disclosed so far.

Although programmed cell death is considered an essential process in every organism, its role in mammals is especially evident in the immune system and in

particular in the thymus (Hedrick et al. 2010; Quaglino et al. 2000), that represents one of the best models for eco-immunological considerations about the ontogeny of the immune system.

In the adaptive immune system, in fact, the pool of mature T lymphocytes is finely modulated by a careful balance of cell survival, death, proliferation and migration to the periphery. Genes activating the autophagy machinery have been demonstrated in T lymphocytes, above all primary CD4+ and CD8+ T cells (Virgin and Levine 2009), suggesting that, depending on cytokine availability, this type of cell death may facilitate both survival and death signalling (Pua and He 2007).

By contrast, apoptosis plays a vital role within the thymus by shaping the T cell repertoire and by deleting unproductive as well as auto-reactive T cells (King and Ashwell 1994; Minter and Osborne 2003). This TCR selection takes place when thymocytes transit from the double negative to the double positive stage; at this point, most thymocytes express a TCR that cannot recognize self-MHC peptide and therefore up to 90% of these cells undergo cell death (Amsen and Kruisbeek 1998). These events are excellently kept under control by the balance of pro- and anti-apoptotic signalling pathways in a way that thymocytes are constrained to three different fates depending, at least in part, on the strength of TCR interactions with MHC molecules expressed by the antigen presenting cells (APC). In particular, cells can undergo death by neglect when TCR fails to be stimulated by APC or survival signals are lacking possibly due to steroids, namely glucocorticoids, produced within the thymus or delivered to the organ by the endocrine system (Chung et al. 2002). Alternatively, death may occur by negative selection when TCR strongly interacts with MHC, whereas, positive selection may arise favouring cells to become either CD4+ T helper or CD8+ cytotoxic T cell precursors.

Currently, it is thought that thymocytes are destined for demise through the intrinsic pathway unless they are tuned into low-avidity TCR signals that promote positive selection or regulatory T cell development.

Moreover, in agreement with previous scattered observations showing that a consistent number of thymocytes, being TUNEL negative, may die in the absence of DNA fragmentation (Nakamura et al. 1995), it has been recently suggested that alternative forms of cell death, as necroptosis (Wu et al. 2012), may actually take place within the thymus.

Due to these stringent control mechanisms, the percentage of mature T cells that survive and migrate from the thymus to the periphery is about 5%, reaching highest values in childhood and progressively diminishing throughout life. Despite the reduction of true thymic size during the age-related involution, there is evidence of T cell output at old age, leading to the assumption that thymocyte development is normal. However, since thymic emigrants from the aged thymus are functionally less responsive, it has been also suggested that, in the elderly, generation of naïve T cells is impaired (Aw et al. 2009).

Even though the characterization of thymic maturation and T cell selection outside mammals is much less characterized, it is worth mentioning the observation that thymic involution is almost the rule among vertebrates. Interestingly, the thymus is subjected also to seasonal variations in both hibernating mammals and cold-blooded amniotes (reptiles) (Zimmerman et al. 2010), but the role of programmed cell death in this process is not well-known. In the frog *X. laevis*, cultured

thymocytes present a high spontaneous percentage of apoptosis, probably due to the absence of pro-survival factors. Moreover, in thymus-derived lymphocytes, apoptosis is induced by corticosterone (Barker et al. 1997) and the involvement of caspases has been observed in the thymic involution induced by glucocorticoids and sexual hormones in the lizard *Hemidactylus flaviviridis* (Hareramadas et al. 2004; Hareramadas and Rai 2005, 2006).

As a whole, it is evident that programmed cell death is intrinsically linked with thymus development and functions in all vertebrates. In this sense, thymus, being an organ generally subjected to a time-regulated involution, could represent a paradox considering that energy is preferentially distributed to organs for immediate physiological necessities. Vertebrates include both short-lived and long-lived organisms, and therefore it is not possible to find a connection between thymic energy expenditure and life span. Never the less, in all these animals, the natural selection seems to have favoured an initial high cost for the evolution of defence mechanisms, i.e., a pre-fixed constitutive cost (Van Boven and Weissing 2004). Studies are still necessary to establish if the programmed cell death observed during thymic involution is linked to energy saving or to avoid damage that could derive from the persistence of thymic functions. As stated below, programmed cell death seems more involved in acute and transitory T cell depletion and T cell selection, rather than in the global thymic involution.

6.5 Thymic Maturation or Thymic Involution: A Process Far Beyond the Concept of Immunosenescence

More than 70 years ago, thymic involution was reported as a process primarily related to aging (Boyd 1932). The mechanisms leading to these modifications have been extensively investigated (Domínguez-Gerpe and Rey-Méndez 2003) and, although changes in stromal cells cannot be ruled out (Hsu et al. 2005), it was suggested that involution could be related to a decreased availability of hormones and/or growth factors (i.e., growth hormone, insulin like growth factor, IL-7) necessary to sustain organ functions. In this sense, the involution process is paradigmatic of the energy redistribution postulated in the trade off conceptualization (Lochmiller and Deerenberg 2000). However, in the case of thymus, the reduction of the allocated energy seems to be related to developmental signals and not to an immediate contingency, such as an immune stimulus.

Results from different experimental models, showed that thymic involution can be actually triggered by many different stimuli, other than aging (Metcalf et al. 1967).

It is known, for instance, that a severe, although transitory, CD4+ CD8+ lymphocyte depletion can be noticed even in the first day of postnatal life and in the following three to four weeks. However, this type of "neonatal involution" seems to be due to a 3 to 4 times higher amount of apoptotic cells, as the result of a prolonged exposure of the foetal thymus to maternal corticosteroids (Raica et al. 2006).

A further condition that may evolve in a non-physiological thymic involution is represented by the response of the organ to injury of epithelial and lymphoid components as it has been also described in patients with acquired immunodeficiency syndrome (Seemayer et al. 1984), although in this case it is secondary to a disease process.

Similarly, the so called "accidental" or "stress induced" involution is frequently in various animals found as a reversible process during seasonal starvation or in case of severe stress due to infections, malnutrition and pregnancy being mediated, at least in part, by the neuroendocrine system through the release of corticosteroids and the involvement of progesterone receptors on thymic epithelial cells. As a result, cortical T cells decrease, whereas macrophages, swallowed up with cell debris and remnants, increase. In some cases, the limit between the cortex and the medulla becomes less defined, Hassall's corpuscles undergo cystic transformation, blood vessels exhibit larger diameters and the amount of adipose tissue increases (Raica et al. 2006).

However, these examples of acute thymic atrophy (leading to complete lymphocyte depletion in only 7 days) may differ from the chronic age-associated involution that is actually characterized by a 3–5 % annual reduction until middle age, when it slows down to less than 1 % per year (by extrapolating these data, in humans the total loss of any thymic structure would occur at the approximate age of 120 years) (Bodey et al. 1997). In the case of acute thymic atrophy, involution is characterized by enhanced death of thymocytes and by the ability to recover after the insult has been removed. In the case of chronic age-dependent involution, it follows a regular pattern for all individuals, though there may be differences between species and sexes (Hsu et al. 2005) and, more importantly, it is not due to increased cell death, but, at least in mammals, to substitution of lymphoid tissue with fat.

Initially, this process was thought to occur at puberty, due to dramatic alterations in the hormonal status, but now it is known that thymic involution begins during or soon after the first year after birth in humans or after few weeks in rodents and continues throughout the entire lifespan (Blackburn and Manley 2004). Therefore it cannot be simply considered an aging phenomenon, but, more likely, the result of tightly regulated mechanisms many of which are still elusive (Aronson 1991; Aw and Palmer 2012). Even within mammals, thymic involution seems to follow different patterns. In humans, the substantial increase in adipose tissue counterbalances the diminution of the original thymic tissue. In mice, the loss of thvmic tissue is not accompanied bv expansion of other tissues, thus causing a net reduction of the organ (George and Ritter 1996). These observations suggest that thymic involution is generally present, but its outcome may significantly differ in various models and for more than one possible reason. However, admitting that energy savings or avoidance of auto-immune reactions could have represented the principle reasons for a positive selection of the trait, a mechanistic stimulus promoting the initial thymic involution remains to be ascertained.

It is well known that life is dependent on oxygen availability, but, at the same time, oxygen may be highly toxic determining the production of reactive species coming out from the electron transport chain within mitochondria (Auten and

Davis 2009). Therefore, organisms have developed efficient mechanisms capable to extinguish these molecules as soon as they are formed (Sen 1995). Never the less, reactive oxygen species (ROS) can be released, thus exerting many biological functions, some detrimental, but some other indispensable in order to modulate cell behaviour and metabolism (Marchi et al. 2012; Sardina et al. 2012).

It is interesting to note that mitochondria-dependent ROS production may induce, for instance, the activation of nuclear factor erythroid-derived factor 2-related factor 2 (Nrf2), that, beside red blood cells, is also present in macrophages where it serves as a transcription factor for xenobiotic detoxification, antioxidant response, as well as a key regulator of early events during adipogenesis (Hou et al. 2012).

At least in humans, but possibly also in other mammals, it can be speculated that, soon after birth, the exposure to increased oxygen levels, may trigger, within the thymus, a shift towards adipocyte generation and fat substitution. Although in the thymus of the DBA/2 mouse model it has been shown that oxidative stress is related to increased peroxiredoxin levels that can initiate apoptosis and thymic involution (Hsu et al. 2005), never the less, other factors and/or stimuli cannot be excluded in order to understand why this phenomenon is so evident within the thymus, despite the widespread occurrence of alteration in the redox balance. The transcriptional mechanisms by which signaling molecules constrain adipocyte differentiation are largely unknown, however, during early adipogenesis, acetylation of histone H3, together with the enrichment in peroxisome proliferator-activated receptor gamma2 (PPARgamma2), CCAAT/enhancer-binding protein beta (C/EBP-beta) and glucocorticoid receptor (GR), were demonstrated. Many of these mediators are potential target of ROS, and the occurrence of epigenomic differentiation from stromal mesenchymal cells towards adipocytes could be hypothesized (Steger et al. 2010). Why adipocytes? For instance, it could be more feasible to directly dispose the organ, once its functions have been completely exerted. For long time, fat accumulation has been considered a possible deleterious side effect of altered and/or inefficient cell metabolism, adipocyte only representing a passive storage of energetic substrates. Now it is becoming always more clear that adipose tissue has a great plasticity and, with its newly discovered endocrine functions, it represents a precious source of many molecules such as leptin, adiponectin, TNFα, IL-1β, IL-6, TGF-β, serum amyloid-A, C-reactive protein, just to mention some of the most important (Trayhurn and Wood 2004; Ronti et al. 2006). These adipokines have a broad range of biological activities, thus influencing not only lipid metabolism and energy balance, but also angiogenesis, blood pressure, inflammation, acute phase response and immunity.

Moreover, it has been recently demonstrated that thymic adipocytes produce angiogenic factors (e.g., vascular endothelial growth factor A and B, angiopoietin 1, and angiopoietin receptor) that, favouring human endothelial cell proliferation and migration, may be able to partly surrogate immune functions (Tinahones et al. 2009) or, at least, to affect thymic functions (Salas et al. 2009). In this way, mammalian thymus it would undergo a reduction in energy investment for immune maturation, but it would remain a potential co-operator of the immune system throughout life.

These observations further support the concepts that (i) immunosenescence cannot be seen as the main consequence of thymic involution since reduction in thymic activity is a natural predetermined process that starts relatively early in life, well before any clear evidence of immunological failures and (ii) atrophy is not realized simply by removing lymphoid cells though programmed cell death, but is achieved by a regulated substitution of lymphocytes by adipocytes, i.e., cells with specific functions possibly allowing to maintain less specific defence mechanisms such as the inflammatory response.

6.6 Why Changes in Thymus Structure and Function Might be Only Apparently Detrimental?

Involution of the thymus has been compared to similarly regressive processes during the ontogeny of holometabolic insects (Jochova et al. 1997; Kaiser et al. 2000), where organs, that have no more functions or are becoming redundant, are readsorbed by programmed cell death, as apoptosis and autophagy (Zakeri et al. 1996; Malagoli 2008; Nezis et al. 2010). As extensively discussed in previous paragraphs, programmed cell death is an unavoidable requirement for the whole immune system, and in particular for the thymus, in order to maintain tissue homeostasis, to impress stringent quality control mechanisms on the pool of T cell migrating to the periphery and to escape pathologic consequences as auto-immune reactions. However, even though these findings can be considered as the foremost purposes of programmed cell death during thymus maturation and aging, it remains to be established the original advantage underpinning thymic involution and if this process has been driven because favoured in terms of energy-balancing.

In addition, it is now well known that in humans the age-dependent involution and atrophy of the thymus are not due to increased cell death, but primarily to substitution of lymphoid tissue with fat.

Thymic atrophy is in fact due to a dramatic loss of the thymic structure (expansion of connective tissue septa, fat accumulation and decrease of lymphoid cells) and function (reduced output of mature T cells and possible less stringent selection process). These events are rather unique within the immune system.

Several questions can be therefore addressed in order to understand why an organ considered to be so important for the immune system undergoes involution in the largest part of the vertebrates studied so far. Many hypotheses have been put forward in order to understand the reasons of these changes (Montecino-Rodriguez et al. 2005; Dowling and Hodgkin 2009; Lynch et al. 2009).

For instance it could be suggested that thymus may not be crucial for the integrity and efficiency of the immune system, although the observation that genetic disorders characterized by thymic aplasia are associated with severe immunodeficiency (Sauce and Appay 2011) underlines the importance of the thymus in the development and maturation of the immune response.

Secondly, it can be proposed that thymic involution is the cause of or it is caused by aging, even though involutive changes start already after the first year of life and well before any evidence of immune deficits and/or alterations.

Thirdly, loss of lymphoid thymic structures could be regarded as the consequence of reduced availability of nutrients and growth factors, since restoring IL-7 levels can rescue aged thymic atrophy (Li et al. 2004; Pesic et al. 2007). Although these changes could play a role in thymic involution, they cannot be considered key pathways triggering an involutive process that, actually, starts quite early, also in the absence of significant alterations in the level of steroids and cytokines. Interestingly, it has been recently suggested that thymic stroma, and in particular thymic epithelial cells, may actively regulate the involution process by down-regulating the expression of specific genes as foxn1 (Chen et al. 2009).

In accordance with the concept of energy trade off, instead of considering thymic involution as a process with detrimental consequences or as the result of failure in tissue homeostasis, it should be regarded as a progressive adaptation of the organ conveying potential beneficial effects to the whole organism (Schuurman et al. 1991; George and Ritter 1996).

Consistently, thymic involution is comprised by a series of events that proceed slowly with time and start after the peripheral T cell repertoire has been established (Quaglino et al. 1998; Capri et al. 2000). Reduced thymic output can be tolerated for a while without any dramatic effects on the function of the immune system, since naïve T cells are long-lived and can undergo homeostatic proliferation in order to keep rather stable the peripheral T cell number (Shanley et al. 2009).

In the light of this hypothesis, it could be suggested that thymic involution takes place in order to conserve resources that become more precious at older age (Aronson 1991, 1993) or that become important in the immediacy, in case of physiological requirements (Lochmiller and Deerenberg 2000). As mentioned before, in an evolution framework, pressure due to increased exposure to a broader range of potentially life-threatening noxae forced organisms to increase the complexity of their defence mechanisms and to build up a composite immune system comprised of many organized structures, including the thymus. Since the large majority of metazoans survive in every environment with the only contribution of innate immune system (Ottaviani et al. 1998), the real advantage in terms of energy efficiency represented by acquired immunity remains to be elucidated (Malagoli and Ottaviani 2010).

The immune system, ensuring the tools necessary for the whole organisms to maintain their homeostasis and to counteract deleterious noxae, has to remain active across lifespan, although at high physiological costs (Ottaviani et al. 2008). This is particularly true in the case of the thymus, that, in order to guarantee the production of T cells with adequate variability, but with precise specificities, set up a series of selection procedures that reduce the effective output of cells to approximately less than 10 % of all generated T lymphocytes. In the perinatal period, the thymus increases in size and output because the peripheral immune system has to be populated, and an optimal repertoire balancing diversity and precursor frequency needs to be established. In this way, individuals are preserved, during their early life,

from tissue damages due to infections and from long-term consequences of fighting against pathologic processes, that would switch resources from growth and development. Consistently, there is an increasing amount of evidence for a direct link between reproductive success and immunoprotection starting from invertebrates up to vertebrates (Holmes and Austad 2004; Cotter et al. 2010).

However, after these thymic tasks are fulfilled and the activity of the organ is no longer stringently required, thymic lymphoid areas are progressively substituted by adipocytes, allowing the subsistence of long-lived lymphocytes that can be maintained in a state of quiescence consuming only 3–5 % of the daily energy intake (Dowling and Hodgkin 2009; Shanley et al. 2009). From the point of view of physiological investments, thymic involution may therefore save further resources with advantages in terms of reproduction that, at younger ages, takes priority even over long-term maintenance (Kirkwood and Rose 1991).

Moreover, diminished thymic activity might also lower the risk that, with age, the reduced strength of T cell selection (i.e., programmed cell death) results in the accumulation of errors with potentially harmful effects in later life (e.g., leukemias and/or auto-immune diseases) (Aronson 1991).

However, it seems hard to balance the evolutionary significance of these proposed beneficial effects with the detrimental consequences associated with involution and thymic atrophy, namely increased infectious disease susceptibility and higher incidence of neoplastic transformations, especially because most of these changes take place after reproductive adulthood. In this context, it has to be mentioned that aging, immunosenescence and degenerative diseases are complex phenomena that cannot be simply related to changes in thymic structure and function or to energy trade-offs, but should be considered as the result of interactions between several factors, as energy metabolism, redox balance, neuroendocrine effectors and, last but not least, the ratio between adaptive and innate responses.

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Chapter 7
The New Antigenic Ecospace of the Globalized World and its Impact on the Immune System: The Battleground of Trade-off and Antagonistic Pleiotropy

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Abstract Like almost all vertebrates, humans have developed a complex immune system, which evolved in an environment that prompted the development of sophisticated strategies of antigen recognition due to the necessity to discriminate relatively few pathogens among huge amounts of commensal microorganisms, and tolerate "self" antigens as well as food antigens. The universe of antigens that driven this evolution could be suggestively called "antigenic ecospace". In this chapter, we will discuss how rapid changes in this ecospace due to its "globalization" as a consequence of new population and economic worldwide dynamics can impact on immune functions and eventually on health status of the individuals.

Keywords Aging · Nutrition · Inflammaging · Antigenic ecospace · Gut microbiota

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3_7,

7.1 Immunological Stimuli

It is well recognized that immune system (IS) is not fixed and unalterable but builds up and differentiate after the interaction with the world of antigens. Antigens therefore represent the stimulus needed for proper development of IS. Vertebrates including humans are born germ-free but are rapidly colonized by microbes and acquire a complex intestinal microbiota soon after birth. Most of these organisms are non-pathogenic to immunocompetent hosts in fact, many are beneficial, supplying vitamins for host nutrition and filling the available microbiological niche to limit access to pathogens. Thus, mammalian health depends on mutualism between host and flora. This is evident in inflammatory conditions such as inflammatory bowel disease (IBD) where aberrant responses to microbiota can result in host pathology. Studies with axenic (germ-free) or gnotobiotic animals have revealed that commensal organisms are required for the development of a fully functional immune system and affect many physiological processes within the host. For example germ-free animals showed a reduction in the content of intestinal IgA-secreting plasma cells and a reduction in size and number of lymphoid follicles within the Peyer's patches. The T cell content of the mucosal immune system is also reduced in germ-free animals: particularly the CD4 + cells of the lamina propria and the CD8 αβT cell receptor of the intraepithelial compartment. In addition, in germ-free animals, central systemic lymphoid structures had a hypoplastic structure, with reduced B and T cell content and poorly formed high endothelial venules. Moreover, alterations in many different non-immune systems have also been described in germ-free state. These include effects on body metabolism, electrolyte and fluid handling, vasculature, liver, endocrine system and behaviour. In most cases the detailed mechanisms underlying physical and functional alterations are not yet fully understood. This surprising range of immune and non-immune host changes in germ-free animals, demonstrate that mammalian bodies are powerfully shaped by the presence of commensal microorganisms.

Thus two important concepts are emerging: 1. we cannot stay alone, in terms of commensal microorganisms and in the same time; 2. the antigenic architecture around us is changing. This chapter will deal with this topic and we will discuss the concept of ecological space, briefly named ecospace and the possible consequences of its globalization on human health.

7.2 Antigenic Ecospace and its Coevolution with Humans: The World Globalization

The immune system has evolved over more than 500 million years (Laird et al. 2000; Shintani et al. 2000) to recognize and to clear invading living micro/macroorganisms and their products. In principle, humans had to cope with antigen exposure typical of their environment that as a whole we can define as "antigenic

ecospace". The concept of antigenic ecospace includes the composition of the antigens, the amount and relative proportions of various antigens that humans were and are exposed, their biological origin (microbes, food, etc.) but also their geographical and chronological distribution. The antigenic ecospace has impacted and still impacts on the immune system both at population level, e.g. host life-history evolution, sexual selection and population dynamics, and at single individual level with strategies of energy cost minimization (Muehlenbein and Bribiescas 2005; Ottaviani et al. 2011).

From about 125,000 years ago, by the use of fire, *Homo sapiens* introduced substantial changes in his living environment and lifestyle. These changes became dramatic when humans from almost isolated hunter-gatherer nomads, became sedentary and farmers and breeders, thus modifying completely their lifestyle. While this got an explosive effect on the nativity giving rise to a substantial increase in the number of the persons, it gave rise on the other side to an unprecedented exposure to brand new antigens due to the close vicinity of cattle and to the changing of food composition, as well to the possibility of spreading out of epidemics, due to the aggregations of people in cities and villages. All these lifestyle/environmental changes in the antigenic ecospace made the immune system to be continuously exposed to infectious organisms and harmless environmental antigens much more than during the whole previous period of mankind history, as far as we know.

In historical times, massive migrations of population, and intercontinental travels leading to the colonisation of America and Australia, represented one of the best known and more dramatic examples of microbial globalization. The diffusion of new viruses and antigens affected the ecospace of previously isolated, autochthonous populations.

After the industrial revolution, technological changes and medical knowledge introduced in many affluent Countries drastically once again altered the exposure to infectious organisms and harmless environmental antigens. These changes have profoundly impacted the function of the immune system and enhanced the development of allergy. This is the base of evidences that support "the hygiene hypothesis". As originally proposed, the hygiene hypothesis (Strachan 1989) postulates that the absence of frequent microbial infections during infancy and childhood, because of lack of exposure to microorganisms, has led to the increase of allergic diseases in recent decades. Allergic diseases, mediated by Th2 response, emerged in the early nineteenth century among people of the wealthy classes and have gradually become more prevalent over the past two centuries (Jackson 2001) in affluent Countries, while in low-income Countries, Th1 response antigens are predominant. "The hygiene hypothesis" was further refined, since exposure to infectious organisms involves more than just microbial infections and longer periods than just infancy and early childhood (Chang and Pan 2008). After its first release the hygiene hypothesis was supported by a vast bibliography. The key players are the T regulatory (Treg) cells that modulate immune reactivity and inflammation. Quantitative and qualitative alterations of such cells imply an alteration of inflammation, leading toward autoimmune processes (Sakaguchi et al. 2009.). The human immune system has coevolved with many pathogens, including intestinal saprophytes, to which immune

system is tolerant (Rook 2009). Many receptors, including toll-like receptors, expressed on immature dendridic cells are stimulated by antigens on the tolerated pathogens/saprophytes, and this interaction stimulate the maturation of dendritic cells that in turn promote the Treg cells-mediated responses to these organisms. This mechanism is a key regulator of the basal inflammatory tone and of the homeostasis of the immune response. The new hygienic condition in industrialized countries has consistently reduced the number of these antigenic interaction and consequently has also reduced the stimulation of Treg cells leading to a disruption of the homeostasis of the immune response and to alterations of the basal inflammatory tone, opening the door to the increased incidence of autoimmune pathologies (Sironi and Clerici 2010).

The host-pathogen interaction has profoundly shaped the genetic evolution of our species. One of the most persuasive examples is the prevalence of thalassemia in Mediterranean regions, as result of the selective pressure of malaria infection. While plasmodium, the pathogen responsible for malaria, has produced a clear mark of its presence, other pathogens may have left signatures that are more difficult to highlight, and probably many other deleterious (or not) alleles are present in the population's gene pool as result of adaptation to specific infectious pressure. (Pozzoli et al. 2010). Genes involved in immunological processes are more exposed to the selective pressure of pathogens, indeed comparative genomic studies has clearly showed that such genes are less conserved and frequently targeted by positive selection than genes involved in other pathways (Kosiol et al. 2008; Sironi et al. 2005). The evolutionary dynamic nature of immune system is also described by population genetics observations. The Wellcome Trust Case Control Consortium studies, where several thousands of individuals where genotyped by GWAS (genome wide association study) methodologies, reported that the genomic regions showing highest levels of population sub-structure (i.e. that are more differentiated between populations, as results of higher evolutionary rate) are the major histocompatibility complex (MHC) cluster and the toll-like receptor 1 (The Wellcome Trust Case Control Consortium 2007). The MHC is one of the best-known examples of balancing selection in humans, indeed its extremely high level of heterozigosity is maintained by pathogen driven selective pressure (Prugnolle et al. 2005). The TLR6-TLR1-TLR10 region on chromosome 4p14 is characterized by high levels of heterozigosity and high levels of nonsynonymous mutations with minimum allele frequency (>0.10) in Caucasian populations with clear marks of positive/balancing selection. The explanation of such selective pressure is that TLR1 receptor in an heterodimeric combination with TLR2, recognizes lipopeptides from Mycobacteria, the causes of leprosy and tuberculosis (Todd et al. 2007). These observation indicates that genetic diversity allows increased flexibility in the immune response and that immune response is always struggling to reach an effective equilibrium between fighting invaders and the tolerance of self and innocuous antigens (Sironi and Clerici 2010).

Taken together all these clues strongly support the hypothesis that the host-pathogen struggle is one of the most demanding challenges that human being had faced during its evolution. This interaction has led to the development of a vast arsenal of sophisticated weapons to fight a vast repertoire of pathogens. The drastic

reduction of this repertoire, that in the last century is recorded in western Countries, has created a new environment that the immune system is not adapted to. While in the developing Countries the host-pathogen interaction is still challenging humans and infectious disease accounts for about 48% of deaths among people younger than 45 years (Kapp 1999), in the industrialized world the radical mutation of the human environment and the evolution of the health care system resulted in a drastic reduction in the of microbial species that we usually deal with. This new environment on one side is one the major contributor of the increase of lifespan in western countries, but on the other side the consistent reduction of the immune system exposure to antigens, has in turn favored the development of chronic inflammatory conditions and autoimmune diseases (Strachan 1997).

In recent times, human migrations became an unprecedented mass phenomenon, due to socio-economic revolutions as well as to increased capacity to move (airplanes, ships, etc.). The persisting migrations of people, especially from low-income to affluent Countries, bring to a sort of globalization not only of economy but also of antigenic ecospace. Thus, the profound social, economic and cultural changes occurring with unprecedented speed from the beginning of the XX century until today, have shaped our way of life. Industrialization and globalization, favoring mass production and facilitating global diffusion of goods and lifestyles have improved people living standards and services worldwide. However modernization and other social changes have led to a more sedentary lifestyle, development of occupation demanding less energy expenditure, and a deleterious dietary habits (diet rich in fat and protein and poor in complex carbohydrates), able to increase lipid accumulation and to favor the development of obesity, insulin-resistance and diabetes (Greenberg and Obin 2006). What will be the final effects of such a globalization on humans? We will speculate on this topic in the final paragraph.

As far as the single individual is concerned, the lifelong interaction with the antigenic ecospace, starting from early life events, seems to be critical for determining individual life span, susceptibility to diseases and successful aging. Humans lives in close association with surrounding microbes and the human body harbor at least 10^{14} microbial cells and a quadrillion viruses constituting our microbiota. In addition, we had to argue that organisms respond to environmental conditions by changing their growth patterns and metabolism. Such changes contribute to the adult phenotypes, including susceptibility to diseases and aging, in both humans and model organisms. Clinical and experimental studies suggest that early life experiences affect lifelong risk of metabolic dysfunction through epigenetic mechanisms, and these effects perhaps last for many generations (Gluckman et al. 2008). Thus, it is believed that the final adult phenotype is the result of the interaction between environment – and the antigenic ecospace as well – the genetic background (individual gene variants) and stochasticity, even if the relative weight of each component changes with time (Cevenini et al. 2010). Moreover, an increasing amount of data also suggests that these phenotypes are, at least partially, underpinned by epigenetic mechanisms. Therefore, in case of a mismatch between the environment of the early and later phases of life we could face an increased susceptibility to disease due to an epigenetic pattern inappropriate for the environment (Low et al. 2011).

Recent data obtained in animal models by means of a longitudinal design suggest that prenatal immune activation negatively affects normal fetal brain development and eventually precipitates a number of long-lasting behavioral, cognitive, neuroanatomical, and neurochemical dysfunctions in the offspring when they reach the adolescent and/or adult stage of development. Meyer et al. (2011) have contributed to the "prenatal cytokine imbalance hypothesis", which in its simplest form postulates that enhanced maternal and fetal expression of pro-inflammatory cytokines critically mediates the association between prenatal immune activation and appearance of neurodevelopmental abnormalities (Meyer et al. 2006, 2008; Vuillermot et al. 2010). Thus, the composition of the antigenic ecospace in utero can prime not only the immune system but also the later development of diseases, being often dependent on maturational processes and pathologically manifest only once the offspring reach adolescence or early adulthood. In addition, some interesting observational studies showed that inappropriate nutrition during pregnancy or early infancy has various negative effects on mental, physical health and mortality during childhood, adulthood and at old ages. In particular, data coming from the Dutch Hunger Winter study and the Dutch Potato Famine study (de Rooij et al. 2010; Lindeboom et al. 2010) demonstrate that a prenatal exposure to food shortage increases the risk of cardiovascular disease (CVD), type 2 diabetes (T2D) and may lead to an age associated decline in cognitive functions. Thus, it has been suggested that early life events may influence later child's health, which in turn may affect future education attainment, earnings, adult health and possibly aging.

These results confirmed the mechanism linking early life nutritional conditions to old-age mortality. Further study showed that exposure to severe undernutrition at 11–14 years was significantly associated with a higher probability of developing T2D, and/or peripheral arterial diseases (PAD) at ages 60–76.

The gastrointestinal tract of neonates becomes colonized immediately after birth with environmental microorganisms, mainly from the mother. Strong evidence suggests that the early composition of the microbiota of neonates plays an important role for the postnatal development of the immune system. The intestinal flora of Caesarean section (CS) and vaginally delivered infants appears to be very different; the former being altered and characterized by a substantial absence of Bifidobacteria sp., the latter characterised by subject-specific microbial profiles, although predominant groups such as *B. longum* and *B. catenulatum* could be identified. Thus, mode of delivery does affect the early stage of intestinal bacterial colonization, which is altered in CS-delivered infants compared with vaginally delivered infants, with only a minor influence of the type of feeding (Biasucci et al. 2008; 2010). The importance of gut microbiota (GM), on human health is dramatically emerging. This endogenous ecosystem, together with the external antigenic ecospace, is coming out as a crucial driving force of the homeostasis of the immune system, and GM lifelong changes, from neonates to centenarians, can represent an important source of inflammatory stimuli (Biagi et al. 2011).

The main thesis is that early events in terms of antigenic ecospace, immune system activation and nutritional conditions may interact with epigenetic factors and likely affect health at older ages. In addition, the first encountering and recognition

of the antigen raises up an immunological process able to respond or not: a short up date of this pivotal aspect will be described below.

7.3 Recognition and Response to Antigens

IS cells use three different strategies to discriminate self and non-self molecules. The best known is that based on the specific recognition of antigenic structures through very sensitive receptors such as the B cell receptor and the T cell receptor expressed on the surface of B and T cells, respectively. The other two strategies are constituted by lack of inhibitory signals represented by normal MHC molecules and recognition of invariant molecular patterns associated with microbes (also called PAMPs). These latter strategies are characteristics of the cells that are considered as belonging to the branch of "natural immunity", such as NK cells and macrophages. While NK cells mainly exert a cytotoxic action upon recognition of their targets through a complicate balance of positive and negative signals generated by the different families of NK receptors (KIR molecules, Leucocyte Immunoglobulin like- Receptors or LIRs, heterodimers of C-type lectins, CD16, NKp30, NKp46, etc.), the recognition of PAMPs by macrophages and other cells, like dendritic cells, causes a more broad spectrum of responses that goes from the induction of the antiviral state to the phagocytosis of the microbe and the production of cytokines and co-stimulatory molecules that serve as activators of the acquired immunity. In this perspective, the cross-talk between innate and acquired responses is becoming the object of more and more intensive studies, as it appears that, according to the model proposed by Matzinger (2002) the specific response to an antigen by lymphocytes is effectively triggered only if this antigen is perceived by the immune cells within a context of "danger", i.e. the sensing of a series of molecules associated to cell damage, indicated as DAMPs (damage-associated molecular patterns). These molecules are indeed released by damaged or stressed host cells and includes high mobility group box-1 (HMGB1), S-100 proteins, heat-shock proteins (HSPs), hyaluronan, surfactant protein, interferon-alpha, uric acid, fibronectin, beta defensin, and cardiolipin (Asea et al. 2002; Biragyn et al. 2002; Lotze and Tracey 2005; Okamura et al. 2001; Peitsch et al. 1988; Seong and Matzinger 2004; Shi et al. 2003; Termeer et al. 2002; Wallin et al. 2002; Wang et al. 1999). Recently, also mitochondrial DNA has been recognised as a DAMP when released outside the cells and can trigger an inflammatory response (Zhang et al. 2010). These molecules can be perceived by the same receptors of the innate immunity that recognise PAMPs, thus building up the cross-talk between the two branches of the immune system. Innate immune cells like macrophages detect the presence of DAMPs and produce cytokines that promote the full activation of the lymphocytes nearby. The fact that, as far as we know, the receptors for PAMPs and DAMPs are largely overlapped is quite puzzling and seems to indicate that: (1) the discrimination self/non-self is probably slighter than previously thought and that recognition of self molecules plays an important role in turning on correct immune responses, and thus it is not always detrimental

as believed in the classical view of the so-called “horror autotoxicus”; (2) these receptors other than recognising foreign structures have a role in directing the localisation of cells of the innate immune system in specific tissues where damage is present in order to recruit and elicit the appropriate cells and responses (Matzinger. 2007). This new vision of the immune response towards antigens largely overcomes the past one according to which they were divided in immunogenic and tolerogenic. According to this new version, tolerance occurs as a consequence not only of the presence of suppressive mechanisms, but also of the anatomical site where antigen recognition occurs.

In this perspective, the most interesting organ to look at is the gut, where the two largest sources of antigens meet each other: food and intestinal microflora. In this environment, an uncountable number of symbiotic and commensal organisms, as well thousands of different alimentary antigens have to be tolerated by our immune system and the (relatively few) pathogens must be discriminated. Therefore, it is likely that alterations of both the gut microflora and gut-associated lymphocytes can modulate the immune response towards the resident antigens, by increasing oral intolerance or lack of control of intestinal pathogens, eventually impinging upon health status of the individuals.

Accordingly, it has been reported that a modification of GM towards a decrease in lactobacilli is associated to frailty in old people (van Tongeren et al. 2005) and that, on the other side, centenarians are characterised by a peculiar composition of the GM (Biagi et al. 2010). Among the cells responsible for the maintenance of such a delicate equilibrium able to discriminate pathogens among the huge plethora of food antigens and commensal bacteria, a crucial role is played by regulatory T cells (Treg). It is known that Treg cells can be induced in the gut, where the availability of TGF-beta, IL-6 and retinoic acid can switch the development of naïve T cells towards Treg or Th17 cells (Weaver and Hatton. 2009). An alteration of these the production of cytokines similarly to what happens during aging process, can have potentially deleterious effects on the gut homeostasis. In animal studies, it has been proven that an alteration in the composition of immune cell populations of the gut is present in the aged mice (Santiago et al. 2011; Gomes-Santos et al. 2012) where a decrease in the frequency of lymphocytes of the intestinal epithelia with regulatory phenotype has been observed, together with a reduced production of TGF-beta and IL-10 in the small intestine. In addition, the ability of dendritic cells from mesenteric lymph nodes of aged mice to stimulate TGF-beta-dependent immune responses was also impaired, even if adaptive CD4 + CD25 + Foxp3 + regulatory T cells at mucosal sites resulted unchanged.

7.4 Gut Microbiota, Aging and Nutrition

The majority of the microbes colonizing our body lives in the intestinal tract. The importance of intestine and food in human health was recognized since 400 B.C. by Hippocrates who said: “Let food be your medicine and medicine be your food” and

"bad digestion is the root of all evil". Microbiota is able to harvest and store energy from food and to perform metabolic functions such as fermentation and absorption of undigested carbohydrates. Since the microbiota colonizes virtually every surface of the human body exposed to the external environment, changes in the surrounding environment will impact on the composition and function of GM. Diet is one of the major determinants for the persistence of a given microbiota in the gastrointestinal tract (GIT), representing the source of nutrients not only for the host but also for the bacterial community. Human studies indicate that changes in dietary habits are able to modulate the gut microbial community. In fact, it has been demonstrated that shifting from high fat/low fiber diet to a low fat/high fiber diet caused remarkable changes in the GM within 24 h and correlates with enterotype (Wu et al. 2011) and that a diet supplemented with chickpeas was able to decrease pathogenic, putrefactive and high ammonia-producing bacterial species, and to increase the butyrate producer, such as the bacterium *Faecalibacterium prausnitzii* modulating the intestinal microbial composition and promoting intestinal health in humans (Fernando et al. 2010). Moreover, a large-scale dietary intervention study determined the effect of the amount and type of dietary fat and carbohydrate on faecal bacteria and SCFA concentrations in people 'at risk' of metabolic syndrome (MetS). The study showed that high carbohydrate diets, irrespective of glycaemic index, may modulate human faecal saccharolytic bacteria, including bacteroides and bifidobacteria. On the contrary, high fat and high saturated fat diets reduced bacterial numbers and increased excretion of SCFA, suggesting a possible compensatory mechanism to eliminate excess dietary energy (Fava et al. 2012). Another aspect to take into consideration is that our microbiota develop and change from infancy to old and very old age as we do. Interestingly, the earliest infant stool, named meconium, harbors very low density of bacteria but the gut of babies is rapidly colonized by the microbes they firstly encounter. As previously mentioned the bacterial colonization of the babies's gut is affected by the modality of delivery, from mother's vagina or by CS, influencing immunological functions during the first years of life (Huurre et al. 2008). Babies born vaginally have microbial composition similar to those of their mother while those born by CS harbor microbiota characteristics of skin (Dominguez-Bello et al. 2010). After 1 years of age, due to the use of antibiotics and the introduction of solid food, the GM of babies changes and become different from those found in the mother and, the phylogenetic diversity increases gradually over time (Koenig et al. 2011). From 2.5 years of life the bacterial composition of babies completely resemble the adult-like microbiota remaining typically stable until old age. Biagi and colleagues demonstrated that young and elderly subjects had a very comparable overall structure of the GM, with *Bacteroidetes* and *Firmicutes* highly dominant (contributing for approximately 95 % to the microbiota), and smaller fractions of *Actinobacteria* and *Proteobacteria*. On the contrary centenarians, subjects who reached the extreme limit of human lifespan, showed, compared to younger people, a significant different microbiota organization and a lower diversity in terms of species composition. In particular, the GM of centenarians showed specific changes in the relative proportion of *Firmicutes* subgroups, an increase in *Proteobacteria*, a group containing bacteria recently redefined as "pathobionts", a decrease in

Faecalibacterium prauznitzii and relatives which are symbiotic species with reported anti-inflammatory properties (Biagi et al. 2010). Gut microbiota play a central role in the development of mucosal immunity since it represent the largest surface area in contact with the ecospace and since it accounts for the largest proportion of the antigens presented to the resident immune cells (Rakoff-nahoum et al. 2004). The human gut associated lymphoid tissue (GALT) maintains the intestinal microbiota under control by a constitutive low-grade physiological inflammation that is based on the network of positive and negative biological feedback processes.

The diminished mucosal tolerance or the age-related changes in the GM composition, or both might favor an abnormal activation of the immune response to the GM components sustaining an inflammatory process. In addition, the nutritional deficiency and age-associated tissue weakness and injuries, common features of elderly subjects, can contribute to trigger a pathogenic inflammatory response in the presence of normally harmless symbiotic bacteria. Furthermore, the decline of IS reactivity and the low-grade pro-inflammatory status characteristic of aging, may advantage pathobionts expansion fueling the inflammation process in a sort of self-sustained loop. It is also possible that the reduced bacterial excretion, due to the slower intestinal transit, fecal impaction and constipation, other frequent features of elderly people, may result in an excessive 'bacterial load challenge', which is known to be a critical determinant for the production of several cytokines modulating the inflammatory response (Maloy and Kullberg 2008). In addition, several gut bacterial species (belonging to the genera *Faecalibacterium, Bifidobacterium* and *Lactobacillus*) known to decline with age, are able to downregulate the pro-inflammatory response at the level of the gut epithelium (Van Baarlen et al. 2009; Biagi et al. 2010; Ouwehand et al. 2008). These bacteria, exhibiting powerful anti-inflammatory properties, may be able to restore the unbalanced cytokine production occurring during aging, providing a rationale for their use as probiotics (Isolauri et al. 2001).

A recent work provided a deeper view on the correlation between the GM composition and the levels of several serum inflammatory markers in humans (Biagi et al. 2010). This study showed that the increase of plasma pro-inflammatory cytokines correlated with changes in the GM profile in centenarians. In particular, the increase of IL-6 and IL-8 was linked with an enrichment in *Proteobacteria* and a decrease in the amount of some butyrate producing bacteria, such as *E. rectale, E. hallii*, and *E. ventriosum*. Thus, it has been hypothesized that the age-related proliferation of pathobionts could either contribute to inflammaging or be promoted by the systemic inflammatory status (Biagi et al. 2011).

Several evidences demonstrated that microbiota, modulating the energy harvest from the diet, energy storage as triglyceride and energy expenditure through fatty acid oxidation, is able to influence the susceptibility to some metabolic diseases such as diet induced obesity, diabetes and IR thus, having a major role in health and disease. In fact, consumption of low glycemic-index and low-energy-dense foods like legumes is inversely associated with the prevalence and incidence of obesity, cardiovascular disease, T2D, some type of cancer and with inflammatory markers

such as CRP, TNF-a and IL-6 (Trinidad et al. 2010; Villegas et al. 2008; Kolonel et al. 2000; Musso et al. 2011, Esmaillzadeh and Azadbakht 2012).

Another metagenomic study comprising 154 individuals (monozygotic and dizygotic twins concordant for leanness or obesity and their mothers) showed that obesity was associated with a reduced bacterial diversity and that "inheritance" of the GM may be more important for microbial community structure and function than the actual genetic context of the host (Turnbaugh and Gordon 2009). Obesogenic dietary patterns may predispose to metabolic diseases and a deviant GM might mediate these events. Metabolic pathways are functionally integrated with immune responses at specific tissue/organ levels, such as liver. Toll-like receptor 4 (TLR-4) play a relevant role in the onset of high-fat diet induced IR as confirmed by the finding that the deletion of TLR-4 prevent this metabolic alteration (Shi et al. 2006). In addition, high-fat, high-carbohydrate diet induce plasma lipopolysaccharide (LPS) elevation and increase TLR-4 and nuclear factor-κB (NF-κB) expression in mononuclear cells promoting IR by increased levels of adipose (TNF)-α and (IL)-6 (Anderson et al. 2007). These increases were totally absent after an American Heart Association (AHA) meal rich in fiber and fruit (Ghanim et al. 2009). Taken together, these data support the concept that endotoxinemia may play a key role in the pathogenesis of obesity and IR and that food ingestion affects plasma endotoxin whose increase may derive from enhanced LPS production by microbiota or from increased intestinal LPS absorption. It is clear that different nutrients have different abilities to induce an endotoxinemic and inflammatory response. Today, due the large use of antibiotics in infants a decreased number of the anti-obesogenic *Bifidobacteria* and *Bacteroides* was observed, contributing to the development of an obese phenotype. Mammalian intestinal Lactobacilli and *Bifidobacteria* have positive effects on obesity in fact they can synthesize from free linoleic acid bioactive isomers of conjugated linoleic acid, which have antidiabetic, anti-atherosclerotic, immunomodulatory, and anti-obesity properties (Terpstra 2004).

7.5 Inflammaging and Immunological Biography

The continuous and long-life exposure and recognition to antigenic load leads to a profound remodeling of the immune system during aging, a phenomenon called immunosenescence, and to the development of a chronic, low-grade inflammatory status termed by our group inflammaging (Franceschi et al. 2000).

Immunosenescence must be considered a dynamic process, and chronic antigenic stimulation, oxidative stress and other harmful agents are major players in this composite lifelong immune remodeling scenario (Franceschi et al. 1998; Larbi et al. 2008). Inflammaging contributes to the pathogenesis of major age-related chronic diseases (atherosclerosis, type 2 diabetes, and neurodegeneration) and geriatric conditions, (sarcopenia, frailty, and disability) involving not only immune cells, but also cells from other tissues and organs of the body, such as adipose, muscle, brain and liver tissues, as well as GM. Considering the co-evolution of GM and host

over millions of years is not surprising that our immune system and particularly the mucosal immune system have established intricate connection with our microbiota.

The antigenic stimulation occurring early in life may impact on the rate of aging of IS and other organs in the body later in life (Deverman and Patterson 2009). The overall efficiency and homeostasis of the immune system likely depends, among other things, on epigenetic modifications of genes involved in immunity and inflammation.

Major open questions at present regard to what extent the history of infections of each individual can modify the epigenetic patterns of the genes involved in the immune response and inflammation, and vice versa how much inflammaging can affect the methylation status of genes involved in basic biological pathways crucial for longevity such as the IGF-1/insulin pathway. Indeed, immune/inflammatory responses are deeply interconnected with nutrition and growth, and are accompanied by concomitant neuroendocrine and metabolic responses (Ottaviani et al. 2008). This complex set of integrated mechanisms is crucial for the maintenance of body homeostasis and is highly conserved throughout evolution.

Thus, the concept of "Immunological biography" emerges and assumes even more importance to understand the etiology of complex age-related diseases. The individual immunological history (ontogenetic time), is probably the best scientific approach to study the epidemiology, the onset and progression of age-related pathologies with a strong immune-pathological component (atherosclerosis, diabetes, dementia, among others) and autoimmune diseases. To this regard, theoretical approach becomes necessary to better focalize and explain complex mechanisms such as human aging and age-related diseases: in this scenario antagonist pleiotropy is taken into account.

7.5.1 Antagonistic Pleiotropy

A classical theory of aging, i.e. the accumulation of unrepaired damages theory postulates that aging is the result of the accumulation of damaged molecules/genetic random mutations, respectively. There are no, or scanty, evidence that this occurs in the IS, as lymphocytes are usually not very long living cells. On the contrary it is possible that a pleiotropic effect takes place. In fact, IS has evolved to protect individuals until the age of reproduction, while today the average lifespan is 80 years, and an ever growing number of persons can live even to 100 years and more, an amount of time much longer than that predicted by evolutionary forces. It is therefore possible that during this very long period of time, the immune reactions can no longer fit the individual's needs. In particular, it is possible that they result too much strong in terms of inflammation, which is good at young age to get rid of infectious diseases, but, as discussed, may be harmful in old age as it can trigger or sustain inflammatory-based diseases. Other aspects of cell biology also affecting the IS can be considered under the light of antagonistic pleiotropy, for example apoptosis and cellular senescence: they may protect from cancer early in life, but may promote

aging of the organisms. Therefore, it seems that, at least as far as the IS, the antagonistic pleiotropy can be a good theoretical model to explain aging. According to this theory, aging is not a selected trait, but the consequence of alleles fixed in evolution by their reproductive advantage early in life, which exert harmful effects in the post-reproductive period. This dualistic behavior was indicated by Williams as antagonistic pleiotropy (Williams 1957). According to this theory, the somatic decline associated with aging would be an inevitable late life by-product of the activity of gene variants that increase fitness early in life, and that are therefore selected by evolution. As mentioned before, strong inflammatory reactions are helpful in young age, and consistently, inflammatory gene polymorphisms responsible for a higher responder status are largely present in the population (Licastro et al. 2005). On the basis of antagonistic pleiotropy point of view, the genetics of human longevity has been proposed to be largely a post-reproductive one (Capri et al. 2008).

On the other side, the rapid change in the antigenic environment, or ecospace, has made us immunologically unfit, as our IS genes were molded by an environment that does not exist anymore. Therefore, in order to remain maximally fit, our IS must try to reshape and reallocate its resources to face an (evolutionarily) unpredicted environment. This reshaping, or reallocation of resources means that the system must "decide" a trade-off between different or even contrasting goals to be reached in presence of a limited amount of resources. This trade-off seems to exist as far as reproduction and soma maintenance, and it has been proposed that the maximum life span as well as the rate of aging of the different species is accounted for by this trade-off (Westendorp and Kirkwood 1998). This theory of aging has been named the disposable soma theory (Kirkwood and Holliday 1979). We are thus in presence of two colliding phenomena: on one side the antagonistic pleiotropic action of IS genes in old age, and on the other one the necessity of the immune responses to reshape in the best possible way in order to face an antigenic ecospace different from that we evolved with. In the sub-paragraphs two cases of possible mismatch between the actual environment and two very important molecular pathways, namely the mTOR and NF-κB pathways will be presented.

7.5.2 mTOR Pathway

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine-threonine kinase known to be receptive of environmental nutrients and energy status. This sensor appears to be one of the most fundamental regulator of cell/organism life span. An impressive amount of literature pointed out the effects of mTOR pathway activation, tightly linked with Insuline/IGF-1 pathway (Bjedov and Partridge 2011), mediated by the two mTOR complexes, i.e. mTORC1 and mTORC2, which regulate different functions, such as cell growth, proliferation, development, autophagy, innate and adaptive immune responses as well as life span. Dysregulation of the mTOR pathway is frequently observed in various diseases and cancers (Zoncu et al. 2011; Weichhart 2012). On the other hand, many Authors indicate

mTOR signaling down-regulation as one of the main molecular mechanism counteracting aging in evolutionarily distant organisms from yeast to mammals (Bjedov and Partridge 2011). Because of its role in regulating gene expression under low nutrient conditions, the TOR pathway emerged as the prime candidate to mediate extension of lifespan by dietary restriction in many animal models (Bishop and Guarente 2007). Actually, this topic is extremely broad and here we want only to outline some relevant aspects tight related to human evolution and gene/environment interactions. In principle, during human evolution growth control program have evolved under conditions of limited food, but these conditions are no longer prevalent in most of the Western world. Overfeeding may be pathogenic because selection has favored organism responses, which are partly mediated by mTOR, that accumulate and store energy in anticipation of periods of shortage. This translates into aberrant cellular responses when food and energy are plentiful and constantly available. From a clinical perspective, chronic mTORC1 activation contributes to obesity by mediating excess fat deposition in white adipose tissue, liver and muscle; in turn, ectopic fat deposition has a role in the insurgence of insulin resistance, in metabolic disorders and diabete (Dazert and Hall 2011; Zoncu et al. 2011). In addition, mTOR inhibits NF-kB dependent pro-inflammatory cytokine IL-12 production in monocytes and activates STAT3-dependent anti-inflammatory IL-10 production, indicating that mTOR has an anti-inflammatory role (Weichhart et al. 2008). To this regard, mTOR appears to be a good example of trade-off between inflammation and successful aging.

7.5.3 NF-κB Pathway

The NF-kB system, an ancient signaling pathway found in both insects and vertebrates, is the master regulator of the innate immunity. The NF-kB system is in the nodal point linking together the pathogenic assault signals and cellular danger signals and thus organizing the cellular resistance. Salminen et al. (2008) argue that NF-kB signaling appears to be the culprit of inflammaging, since this signaling system integrates the intracellular regulation of immune responses in both aging and age-related diseases and recently our group has studied the NFkB interactome thus dimostrating the impressive amount of relationships among proteins directly or not linked to it.

Moreover, NF-κB is a key regulator of diverse intestinal responses modulating the expression of many genes with important functions in inflammation and regulating innate and adaptive immune responses. Thus, deficiency in or hyper activation of NF-κB results in chronic intestinal inflammatory disorders.

The intestinal microbiota, as above described, profoundly influences health through effects on nutrition, immunity, intestinal epithelial homeostasis and intestinal development. Intestinal microbial regulation of nuclear factor NF-κB signaling is one of the best-studied pathways determining intestinal homeostasis and diseases. Growing evidence suggests that ‘optimal’ NF-κB activity plays a significant role in

maintaining normal intestinal homeostasis. However, hyper-activation of NF-κB results in chronic intestinal inflammatory disorders. In fact, obese and Crohn's disease patients showed a lower presence of *F. prausnitzii* a microorganism that is able to inhibit NF-kB activation (Sokol et al. 2008). As already described in previous paragraphs also centenarians showed a reduction of this microorganism with anti-inflammatory properties contributing to inflammaging (Biagi et al. 2010). On the contrary, some *Lactobacilli* (*L reuteri* and *L salivarius*, for example) are able to suppress TNF-α or S. typhimurium induced IL-8 gene expression and secretion by intestinal epithelial cells in a NF-κB dependent manner (Ma et al. 2004; O'Hara et al. 2006). Moreover, it has been shown that *L. casei* suppressed invasive *S. flexneri* induced transcription of inflammatory cytokines, chemokines and adhesion molecules in intestinal epithelial cells through modulation of the ubiquitin/proteasome pathway to stabilise IκB and thus inhibiting NF-κB nuclear translocation (Tien et al. 2006).

Another mechanism of probiotic regulation of NF-κB transcriptional activity in the nucleus is through activation of peroxisome proliferators activated receptor (PPAR)γ (Kelly et al. 2004). Interesting, a recent paper investigating the metabolic activity of NF-κB, using two different mouse models with elevated NF-κB activities demonstrating that mice were resistant to adulthood obesity and diet-induced obesity without reduction in food intake. The adipose tissue growth and adipogenesis were inhibited by the elevated NF-κB activity and PPARγ expression was reduced by NF-κB at the transcriptional level. The two models exhibited elevated inflammatory cytokines (TNF-α and IL-6) in adipose tissue and serum. These data suggested that the transcription factor NF-κB promotes energy expenditure and inhibits adipose tissue growth, but are not conclusive on mice life span (Tang et al. 2010).

In humans chronic inflammatory status favors the onset of many pathologies and the currently proposed anti-aging strategies are all based on decreasing the inflammatory component-

NF-kB represents, as well as mTOR, another good example of trade-off between inflammation and successful aging but even more it is one the most interesting example of antagonistic pleiotropy.

7.6 Conclusions

As largely discussed in this chapter, IS is a dynamic tissue that changes in composition and functionality all along the life of an individual. As for many other aspects of human biology, the final phenotype (immune responses) is shaped by the intimate relationship between IS cells and antigenic ecospace and it faces from time to time. Since the genes of the IS, as well as all the genes of our cells, have been selected for the best fitness, this means that they are selected to give the better response to the ecospace they evolved with from birth to reproduction. After the age of reproduction, a sort of battle-ground between the trade off of gene variant relevance and antagonist pleiotropy play a role on various genes/pathways regulation.

Since the composition of the ecospace has rapidly changed in the last decades due to the world globalization, it is possible that the interaction with the genes of our IS cells is no longer optimal, in other words we are becoming unfit towards the new antigenic environment, with possible negative consequences on our health. Moreover, since average lifespan is increased thanks to the technological and cultural improvements of our habitat, such an interaction between IS and antigenic ecospace lasts much longer than before, exhacerbating possible unfitness. On the other side, the excess of hygiene has led to the emerging of autoimmune and allergic diseases, therefore it is also possible that a globalized diffusion of antigens could restore the predominance of pro-inflammatory responses we were selected for, thus leading to a decrease in autoimmune and allergic diseases, likely without a correspondent increase in infectious diseases thanks to the help of modern generations of antibiotics and other drugs.

Which of these hypotheses will turn to be true only time will tell, what we know is that a "healthy" development and functioning of our IS needs the interaction with a proper antigenic ecospace, thus what we have to do in the next future is to provide the next generations with an environment in which antigens are diffused (not too much restricted), but not to the point of spreading new diseases. This will be a crucial challenge of the next years and deserve new studies to be tackled properly.

Acknowledgements The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007–2011) under grant agreement n. 259679 (IDEAL) and KBBE 2010–2014 n. 266486 (NU-AGE) to CF.

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Chapter 8
Population Immunology: Germs, Aging and Inflammation

Ana Maria C. Faria and Claudio Franceschi

Abstract Immunosenescence has been described as a combination of dysfunctional immunity with a state of low grade chronic inflammation (inflammaging) in the elderly. These processes may result either in increased susceptibility to infections or in the development of chronic inflammatory diseases and frailty in the elderly. However, not all elderly bear a dysfunctional immunity. Senescence may also be accompanied by a remodeling of the immune system that is associated with increased longevity and healthy aging. The concept of healthy aging has been proposed initially in Europe to describe the individuals who reach advanced age free of inflammatory consequences of immunosenescence. Since then, the quest for biological markers of healthy aging has been focused on the consequences of inflammaging, but this inflammatory status might be a deleterious event restricted to populations living in clean environments. In other parts of the world where infectious diseases are still prevalent, inflammatory responses may be beneficial and needed to cope with infections agents. Therefore, the debate over the biological markers of healthy aging would have to take into account geographical, cultural and environmental differences that reflect the changes in the past century in our contacts with microorganisms and their immunological consequences.

Keywords Aging · Inflammation · Infection · Genetics · Microbiota

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3_8,

8.1 Introduction

Immunosenescence has been described as a combination of dysfunctional immunity with a state of low grade chronic inflammation (inflammaging) in the elderly. Both processes may result in deleterious consequences. Dysfunction in protective immunity may lead to increased risk of infections; a long standing inflammatory state is usually associated with tissue damage and chronic degenerative diseases. However, not all aged individuals develop chronic diseases, frailty or suffer with chronic infections. Indeed, we have observed that, in healthy European centenarians, chronic inflammatory events are less evident and are compensated by immune-modulatory mechanisms. On the other hand, in developing countries, there are individuals living in endemic areas for infectious diseases who stay clear of infection throughout life due to the preservation of robust mechanisms of innate immunity. Therefore, senescence is characterized by either a decline or a remodeling of the immune system. The concept of healthy aging was proposed initially in Europe to describe the individuals who reach advanced age free of the inflammatory consequences of immunosenescence. However, this concept must encompass geographical and populational variations related to the role of the increased inflammatory state of the elderly and to the cultural changes in modern civilization that have altered our contact with microorganisms of all sorts.

8.2 Aging and Inflammaging

8.2.1 Inflammaging

Senescence is reportedly associated with increased susceptibility to infection (Daynes et al. 1993) and progressive decline in immune function (Miller 1996). Several aging-associated immunological alterations are already described in medical literature, mostly in the T-cell compartment. They include involution of the thymus, reduction in the number of naive CD3+ cells with a parallel increase of oligoclonally expanded CD4+ cells with a memory phenotype, reduced potential to produce IL-2 and loss of CD28 expression (Akbar and Fletcher 2005; Malaguarnera et al. 2001; Pawelec and Solana 2001). The most pronounced change in the B cell subset is the reduced production of naive B lymphocytes although immunoglobulin secretion is well preserved (Weng 2006). Therefore, aging is associated with an accumulation of activated T and B cells and a decline in the ability to mount immune responses to novel antigens (Hodes 1995; Pawelec et al. 1998).

In the past decade, a new approach to the study of aging emerged from the data collect from centenarians. These data were obtained using a strictly biochemical and clinical inclusion and exclusion criteria, known as SENIEUR protocol (Ligthart et al. 1984), and they showed that senescence is not necessary associated with

deterioration of the immune function but rather to a remodeling of the immune system. In this sense, many immunological activities are well preserved in the elderly and they may compensate for other functions that are impaired (Cossarizza et al. 1997; Franceschi et al. 2000). The concept of successful or healthy aging comes from these studies and it eliminated the confusion between aging and age-related disorders. Although changes in T and B cell function were identified in healthy elderly, innate immune responses seem to be more resistant to age-associated changes (Cossarizza et al. 1997). Innate immunity is well preserved in senescence and this is reflected in the number and cytotoxicity activity of NK cells that are increased in healthy old people and centenarians (Sansoni et al. 1993; Solana et al. 1999; Solana and Mariani 2000).

In this sense, healthy immunosenescence is a net result of the continuous adaptation of the body to the deteriorative changes occurring over time. According to this hypothesis, body resources are continuously optimized, and successful immunosenescence must be considered a very dynamic process of remodeling (Sansoni et al. 1993; Solana et al. 1999; Solana and Mariani 2000). We would like to propose a novel concept to be added to this hypothesis: that remodeling must be of a distinct type depending on the challenges faced by the elderly.

A remarkable and universal feature of immunosenescence is the filling of the immunological space with memory and effector lymphocytes as a consequence of thymic involution, reduction in the output of naïve T and B cells, and long standing exposure to antigens. The continuous attrition caused by clinical and sub-clinical infections, as well as the continuous exposure to other types of antigens (food, allergens, microbiota), has been considered as responsible for the chronic activation of the immune system and inflammation in frail senescence. In European individuals, this age-related chronic inflammatory activity, inflammaging, lead to tissue damage, to the emergence of inflammatory chronic diseases, and it is related to mortality risk in the elderly (Franceschi and Bonafe 2003). A wide range of age-related diseases, such as neurodegeneration, atherosclerosis, diabetes, auto-immune diseases, osteoporosis and sarcopenia, among others, share such inflammatory pathogenesis (Sansoni et al. 2008). According to this, immunosenescence, morbidity and mortality would be accelerated in those subjects who are exposed to an extra burden of antigenic load. If we consider that aging is also associated with reduction in the expression of some heat shock proteins known to play a role in compensating cellular stress (Rao et al. 1999; Njemini et al. 2003), this continuous accumulation of stress attrition with aging would be particularly deleterious.

Contrary to this situation, inflammatory immune responses may also be needed to cope with infectious agents in other parts of the world where they are plenty and infectious diseases are still prevalent. In that case, it is plausible that inflammatory cells in aged individuals would not be deleterious since they would be preferentially directed against these infectious targets. Activated T cells that undergo large expansion in an environment that is free of infection usually react with either self components or microbiota since these antigens are not only present but they are also abundant as a natural source of stimulation for proliferation (Min et al. 2004;

Wood et al. 2009). Filling up the immunological space in European aged individuals may have consequences analogous to homeostatic proliferation in pathogen-free lymphopenic animals. As a counter example of the same phenomenon, there are several pieces of evidence in experimental models and in human studies showing that infectious conditions, schistosome infection for instance, induce regulatory T cells that modulate inflammatory diseases such as colitis (Moreels et al. 2004), airway inflammation (Yang et al. 2007), experimental autoimmune encephalomyelitis (Zheng et al. 2008), diabetes in NOD mice (Zaccone et al. 2009), collagen-induced arthritis (He et al. 2010), among others. Recent studies correlated Schistosome infection in humans with lower levels of anti-nuclear antibodies (Mutapi et al. 2011), and antigens from this parasite have been proposed as therapeutic candidate agents for autoimmune and allergic disaeses (Araujo et al. 2004; Osada et al. 2011).

The hypothesis we would like to explore is that inflammaging is a particularly deleterious event in populations living in clean environments. In these individuals, inflammatory immune responses are either directed to few highly prevalent infectious agents (such as cytomegalovirus) or to abundant but innocuous antigens such as self components. In both cases, oligoclonal T cell responses may result in tissue damage and frailty in the elderly. In individuals in whom inflammatory responses can be directed to prevalent infectious, they may be advantageous.

8.3 Antigenic Load: a Stressor or a Tonic Stimulus

One of the major differences between invertebrates and vertebrates is the emergence of a clonotypical immune system, characterized by a large repertoire of lymphocytes that provide specificity and memory to the induced immune responses. The vertebrate immune system can be considered a more complex counterpart of the anti-stress response network identified in invertebrates as the major determinant of survival. It can also be seen as a sophisticated mechanism to deal with molecular signals being them non-harmful (food, microbiota, self components) or dangerous antigens (infectious agents). Balancing regulatory responses to the former with inflammatory responses to the later is critical for health, but both types of antigens are able to activate immune cells.

Under this evolutionary perspective, antigens may represent a particular type of stressor (antigenic stress) and immunosenescence can be envisaged as the consequence of the continuous attrition caused by chronic antigenic load. However, since not all antigens (including bacteria) represent threatens to the organism, there might be differences in the long term immunological outcomes (immunosenescence being the most dramatic of them) according to the kind of stimulus they provide.

8.3.1 Role of Microbiota and Dietary Antigens in the Development of the Immune System

The human gut microbiota represents the biggest and most diversified symbiont community of our body. The density of bacteria reaches 10^{12} organisms per gram of stool with more than 1000 different species (Turnbaugh et al. 2007). This vast complex community has a metabolic capacity comparable to that of the liver (Frank and Pace 2008). Two dominant divisions of bacteria are the main populations in the human gastrointestinal tract, *Firmicutes* and *Bacteroidetes,* while *Actinobacteria, Proteobacteria, Fusobacteria* and *Verrucomicrobia* are subdominant divisions. Essential for the hydrolysis of complex plant polysaccharides, the production of short chain fatty acids, the biosynthesis of vitamins and the regulation of fat storage, bacteria from our microbiota increase our capacity to extract energy from diet. In addition, gut microbiota helps to strengthen the gastrointestinal epithelial barrier, and defends the host from colonization by pathogenic microorganisms (Neish 2009; Candela et al. 2010; Ottaviani et al. 2011).

Anaerobic bacteria fermentation in the intestine results in metabolic products with modulatory activity in the gut such as short chain fatty acids (SCFAs) acetate, propionate and butyrate. In addition to their important role as fuel for intestinal epithelial cells, SCFAs modulate different processes in the gastrointestinal (GI) tract such as electrolyte and water absorption. These fatty acids have been recognized as potential mediators involved in the effects of gut microbiota on intestinal immune function. SCFAs act on leukocytes and endothelial cells through at least two mechanisms: activation of GPCRs (GPR41 and GPR43) and inhibition of histone deacetylase (HDAC). SCFAs regulate several leukocyte functions including production of cytokines (TNF-α, IL-2, IL-6 and IL-10), eicosanoids and chemokines (e.g., MCP-1 and CINC-2) (Vinolo et al. 2011).

Moreover, stimulation by microbiota-derived antigens is important for the full development of the adult immune system. Adult germ-free mice have reduced levels of secretory IgA, and an underdeveloped gut-associated lymphoid tissue (GALT). These animals also present systemic alterations such as low levels of serum IgG and IgA (Moreau et al. 1982). Microbiota strongly impact on mucosal B cell physiology and lead to production of serum immunoglobulins due to upregulation of genes and proteins involved in later stages of B cell response (Hansson et al. 2011).

Food components seem to have a similar role in the development and function of the immune system. Several micronutrients, such as zinc and selenium, have been already associated with immunological action. Zinc deficiency causes significant impairment in both adaptive and innate immune responses, and promotes systemic inflammation. It is interesting to note that there are remarkable similarities between the hallmarks of zinc deficiency and immunological dysfunction in aged individuals. Both processes are characterized by impaired immune responses and systemic low grade chronic inflammation. A major target of zinc is NF-kB, a transcription factor critical for the expression of proinflammatory cytokines whose production is regulated by extra- and intracellular activating and inhibiting factors interacting

with the regulatory elements on cytokine genes (Vasto et al. 2007; Wong and Ho 2012). The effect of vitamin A metabolite retinoic acid in the generation of regulatory T cells in the gut is also a recent finding that highlights the critical role of dietary components in shaping our immune system. It has been described that retinoic acid and TGF-beta produced by specialized gut CD103+ dendritic cells can drive the convertion of naïve CD4+ T cells in the gut mucosa into a regulatory phenotype (Mucida et al. 2007; Coombes et al. 2007). Therefore, dietary micronutrients seem to function not only as nutrients but also to play a role in the immunoregulatory circuits of body.

Food proteins are particularly critical in this scenario since they represent a daily load of antigenic materials. We have previously shown that mice fed a diet in which intact dietary proteins were replaced by equivalent amounts of amino acids from weaning (day 21 of life) up to adulthood (12 weeks of age), presented an immunological profile similar to neonates. Adult animals reared in this balanced amino acid-based protein-free diet (Aa-fed mice) grow normally without macroscopic or biochemical signals of malnutrition but showed local and systemic abnormalities in their immune system. Aa-fed mice have poorly developed gut-associated lymphoid tissue (GALT), low levels of secretory IgA as well as decreased levels of serum IgG and IgA. Moreover, T cells isolated from lymph nodes and spleen secrete cytokines of a predominant Th2 profile, resembling neonates (Menezes et al. 2003). Impairment in Th1 responses in these mice and their immature immunological status result in retarded immune response to *Leishmania major* infection (Amaral et al. 2009). All these immunological parameters, however, can be restored to normal levels if the animals were fed a protein-containing diet for 3 days suggesting that the immunological immaturity is not permanent and it is indeed antigen-dependent (Amaral et al. 2006).

Altogether, these data indicates that antigens derived from food and microbiota play a critical role, after weaning, in the development of a mature immune system. Therefore, these natural antigens seem to represent physiological stimuli designed by evolution to shape immunity and to prepare the immune system for future challenges. It is interesting that although pro-inflammatory cytokines, such as IFN-gamma, IL-6 and IL-12, are upregulated in adult conventional mice, a high production of regulatory cytokines is also sustained as to relate immunological maturity relates to a balanced state of lymphocyte activation.

In addition, it is known that oral exposure to antigens by the oral route leads to a state of specific suppression of inflammatory responses called oral tolerance. Animals and humans are tolerant to their dietary antigens as well as to their authochthonous microbiota (Faria and Weiner 2006; Duchman et al. 1997). Induction of immune tolerance and maintenance of the homeostatic balance represent strategies used by commensals. Some bacteria actively suppress immune responses by inhibiting NF-kB activation in epithelial cells. The resident microbiota, by producing TLR-mediated signals, can modulate epithelial and dendritic cells in the lamina propria towards a tolerogenic pattern. Antigen presentation in such context would induce preferencially T regulatory lymphocytes (Tregs) in the gut subepithelium to produce IL-10 and TGF-β. These factors that are major contributors for tolerance

induction by the innate and the adaptive immune systems to the resident gut microbiota (Izcue et al. 2006). The generation of regulatory T cells is a mechanism known to operate in oral tolerance to food antigens that reach Peyer's patches or gut lamina propria (Faria and Weiner 2005).

Oral tolerance is of unique immunologic importance since it is a continuous natural immunologic event driven by exogenous antigens that contact the body via the gut mucosa. The mucosa of the small intestine alone is estimated to be 300 m^2 in humans and there are 10^{12} lymphoid cells per meter of human intestine. Approximately 130–190 g of dietary proteins is absorbed daily in the gut and the number of bacteria colonizing the colonic mucosa can reach 10^{12} microorganisms/g of stool as already mentioned (Mestecky et al. 2007; Candela et al. 2010). Due to their privileged access to the internal milieu, these antigens that continuously contact the mucosa represent a frontier between foreign and self-components. Thus, oral tolerance is a form of peripheral tolerance that evolved to treat external agents that gain access to the body via a natural route as self components.

It is true that aging is usually related to a decrease in susceptibility to oral tolerance induction when certain regimens of antigen administration are tested (Faria et al. 1998) but ingestion of antigen in a continuous feeding protocol that resembles natural conditions of feeding leads to tolerogenic consequences even in senescent mice (Faria et al. 2003).

Although antigenic load may be considered as a major component of the chronic activation of the immune system during aging, it is clear that the immunological impact of food and microbiota components are mostly in the regulatory axis of activation. Moreover, contacts with these two classes of antigens may be considered as a developmental part of immune maturation and, at the same time, part of the construction of an immunological identity.

8.3.2 Hygiene hypothesis—Infections as Beneficial Immunological Stimuli in Childhood

Microbiota and dietary antigens are clearly innocuous natural antigens that would elicit regulatory immune responses, but we cannot list pathogens in the same category. Contact with infectious agents usually leads to inflammatory responses and they may be considered as important stressors that continuously impinge on our immune system. Lifelong antigenic challenges by infectious agents may lead to a condition of chronic inflammation (inflammaging), with increased lymphocyte activation and proinflammatory cytokines (Ginaldi et al. 2005; Martinis et al. 2005). An elderly immune system becomes more and more predisposed to chronic inflammatory diseases and is less able to respond to acute challenges by new antigens.

Since the classical study by Strachan (2000) on family size, infection and asthma prevalence in England and New Zealand, a new concept emerged on the role of childhood infection in creating immunoregulatory mechanisms that would protect us from atopy and autoimmunity. This concept was called "Hygiene hypothesis"

and it provided the strongest epidemiological explanation for the rise in allergic disease in the past decades in western world. Recent literature has extended the epidemiological findings of the protective effect of being born and reared in a farm environment and associates a larger diversity of organisms with protection from allergic and autoimmune diseases. Furthermore, human and animal studies provide increasing evidence for the role of both the innate and adaptive immune systems, including regulatory cells, as mediators of this protective effect (Frei et al. 2012). The timing of exposure to infection and the properties of the infectious agent, in addition to the genetic susceptibility of the host, play an important role in the future development of allergic and other chronic inflammatory diseases.

The hygiene (or "Old Friends") hypothesis suggests that the increase in chronic inflammatory disorders that started in Europe in the past century resulted from lack of exposure to microorganisms that we co-evolved with providing immunoregulatory circuits that shaped our immune system to deal with the chronic load of antigens that naturally contact the body. Probably these childhood common infections have a role similar to that attributed to microbiota antigens. The consequences of the loss of the Old Friends and distortion of the microbiota are aggravated by other modern environmental changes that also lead to enhanced inflammatory responses (obesity, vitamin D deficiency, pollution, etc.). The range of chronic inflammatory disorders affected may be larger than it had been assumed (allergies, autoimmunity, inflammatory bowel disease, but also coeliac disease, food allergy, vascular disease, some cancers, and depression/anxiety when accompanied by raised inflammatory cytokines). (Rook 2012).

Modern societies and industrialization exert a dramatic impact in the microbial colonization of the human gut. They favoured changes in human ecology that strongly reduced the microbial diversity that is associated with frailty in the elderly. Recently, age-related differences in the gut microbiota among young adults, elderly, and centenarians have been explored by quantitative PCR of 16 S genes of Bacteria and Archaea (Biagi et al. 2010). It was found that the gut ecosystem of young adults and 70-year old people is highly similar but differs significantly from that of the centenarians. In this later population, microbiota shows clear signs of reduced diversity with increased frequencies of pathobionts such as Fusobacterium, Bacillus, Staphylococcus, Corynebacterium and many members of Probacteriae. In spite of that, there is enrichment for symbiotic species with reported anti-inflammatory properties, such as *Eubaterium limosum* and relatives, suggesting that alterations in the composition of gut microbiota in centenerains may represent a form of remodeling associated with successful aging.

From the data on the role of natural antigens (food and microbiota) in immune system maturation after weaning and on the impact of infections during childhood in the robustness of the regulatory immune network in adults and old individuals, it is clear that the antigenic load at early time in life has a predominant beneficial effect in the immune system. Since we have co-evolved with an associated microbiota and food components, and since adult life in traditional communities of the past use to extend only until 40–50 years of age, it is understandable that antigenic stimulation in this period has a great impact in the organism. Moreover, the immune system

of a young individual is still populated by a large repertoire of lymphocytes and immunity at this period is characterized by diversity and plasticity. Aging is associated with loss of this plasticity (mainly due to repertoire shaping) and therefore the aged immune system is less capable of dealing with novel antigens. Antigenic load at this late period of life would represent a burden rather than a growth stimulation.

8.4 Genetic Background and Epigenetic Factors as Major Determinants of Immunosenescence

Inflammaging is described as universal phenomenon that accompanies the aging process, and which is, in European populations, related to frailty, morbidity and mortality in the elderly. However, there are people who become frail and suffer early in life from age related diseases that have an inflammatory pathogenesis; others become healthy centenarians in whom high levels of inflammatory mediators are present, thus suggesting that inflammaging is compatible with very old age. A collection of data obtained by our group indicates that these different age-related trends, i.e. inflammaging and frailty, may have strong genetic and epigenetic components.

Estimates of the heritability of human lifespan vary from 10 to 50% with most common finding being that about a third of human lifespan may be heritable. The rest is due to environmental exposure, accidents and injuries, lifestyle and chance (Gravina et al. 2009). Very long life, to beyond the age of 90 years, appears to have an even stronger genetic basis (Perls et al. 2002). Despite the challenges of studying complex traits such as lifespan, studies have reported alleles that were significantly associated with human longevity.

Some of the genetic associations with longevity and aging came from the studies on caloric restriction in animals showing that mitochondrial-derived free radicals produced during ATP production are involved in aging. Studies in several animal species demonstrated a strong correlation between oxidative stress in the mitochondria and life span since diet restriction in rodents increases their life span by up to 50% (Sheldon et al. 1995). Indeed, some of genetic markers of longevity are proteins associated with regulation of the respiratory process in mitochondria called uncoupling proteins. Uncoupling protein 1, 2 and 3 (UCP1, UCP2 and UCP3) belong to a large family of mitochondrial transmembrane carriers. These proteins are able to dissipate the proton gradient of the inner mitochondrial membrane when activated, decreasing ATP-generation. The resulting decreased ROS production has been considered as a link with the “thinness and longevity” phenomenon observed during diet restriction (Andrews and Horvath 2009). Another set of proteins involved in the same class of events are sirtuins. Genetic variations on sirtuins genes (SIRT1, 2 and 3) have been reported as involved in mitochondrial functionality and longevity (Guarente et al. 2005; Lescai et al. 2009).

Several candidate genes related to regulation of cell cycle, and telomere length have also been described as associated with longevity. p21 (Gravina et al. 2009)

FOXO3A, TERT and TERC are examples of such genes. CDKN1A or p21 gene is interesting in this sense because it is a stress-inducible senescence-associated cell cycle inhibitor whose expression is implicated in the upregulation of several age-related genes. p21 promoter is induced by some signals involved in stress response and inflammation (such as TGF-beta, IFN-gamma, IL-6) that contributed to the pathogeneses of many age-related diseases (Carrieri et al. 2004; Olivieri et al. 2007; Franceschi et al. 2007).

On the other hand, many of the genes that have correlated with longevity are associated with susceptibility to age-related diseases (Franceschi et al. 2007). According to James Fries, morbidity and lethal impairment compresses toward the end of life (Fries 2000). Therefore, long-lived individuals in Western countries are most likely the ones spared of diseases such as cardiovascular disease, Alzheimer disease, diabetes mellitus, and cancer. They may either lack numerous gene variants that provide susceptibility to these diseases or they may carry protective variants. One of the most consistent reported associations has been APOE (Apoliprotein E) (Dato et al. 2007), whose variants were found to be linked to increased susceptibility to cardiovascular disease and Alzheimer disease (Corder et al. 1993; Schächter et al. 1994). Several studies have shown that variations in the FTO (Fat mass and obesity-associated protein) gene are also associated with age related diseases. It has been reported that FTO polymorphisms are involved in increased fatness and obesity in humans (Andreasen et al. 2008) and a SNP (Single Nucleotide Polymorphism) in FTO gene is found to be associated with morbidity and mortality through the effect on fatness (Zimmermann et al. 2009). Recently it has been shown that they can also increase risk of Alzheimer's disease dementia (Keller et al. 2011).

Recent studies have revealed that variations not only in genomic DNA variations but also in mitochondrial DNA and micro RNA are implicated in healthy aging and longevity.

It has been recognized for a long time that age-related random damages to mitochondrial DNA (mtDNA) and the consequent decrease in the respiratory chain capacity are among the major contributors to the aging process (Harman 1972; Kujoth et al. 2007; Trifunovic et al. 2007). Tissue-specific somatic mutations in the mtDNA Control Region (CR) can reach high levels in aged individuals (Michikawa et al. 1999; Wang et al. 2001). These mutations occur at mtDNA sites which are critical for replication or transcription, suggesting they can be relevant for the aging process. Interestingly, CR heteroplasmic point mutations are over-represented in centenarians when compared to younger subjects in the Italian population.

A recent genome-wide micro RNA (miRNA) study in centenarians and nonagenarians using a hierarchical clustering analysis of the miRNA microarray expression data revealed a distinct separation between the long-lived individuals and the younger controls. Moreover, p53 and other cancer pathways were shown as potential targets of the down-regulated miRNAs suggesting that they could be involved in the prevention of tumorigenesis and the maintenance of genomic integrity during aging. (El Sharawy et al. 2012).

The role of genetic background in immunosenescence highlights the importance of studies on individuals coming from distinct ethnical groups since each

one would carry immunologically relevant polymorphisms at different frequencies. Moreover, studies on aging, which take into consideration demographic data, suggest that different susceptibility genes are likely to underlie the longevity phenotype in genetically distinct populations. A recent study in Italian centenarians on the chromosomal region 11p15.5, which encloses five genes (HRAS1, SIRT3, TH, INS and IGF2), the variation of which was found to be associated with life extension, did not confirm the earlier findings obtained in German individuals (Lescai et al. 2009). Analyzing the contrasting results from different populations, the authors hypothesized that centenarians (or healthy elderly) might be considered phenocopies who display the same phenotype (longevity), but who have attained healthy aging by taking advantage of different gene\environment contributions (De Benedictis and Franceschi 2009).

8.5 Towards a Population Immunology: The Role of Inflammaging in Different Populations

Inflammaging has been considered so far as universal phenomenon associated with frailty and morbidity in the elderly. Nevertheless, most of the studies supporting the concept were performed in Europeans individuals. Even among these reports, authors have observed variations and contrasting results as their samples vary demographically and geographically. This has been reported for longevity-related genes and also for microbiota analysis in the elderly (Rajilić-Stojanović et al. 2009; Biagi et al. 2010).

On the other hand, regions where life expectancy was not high and centenarians were rare are undergoing important changes in recent years. Developing countries, such as Brazil, India, Mexico and others are experiencing an unprecedented growth in the number of their elderly as a result of several factors including decrease of poverty, improvement in sanitary conditions and better access to medical care (Gazzinelli et al. 2012). The impact of social and public policies in the health and life expectancy is illustrated by the relatively higher social welfare and health levels in some welfare states, such as Kerala in India (World Health Organization 2007). Similarly, significant increases in infant vaccination, school attendance, and pre-natal visits to clinics in Brazil were largely attributed to the family assistance programme “Bolsa Familia” (Family Grant), particularly in the poor northeastern region (International Food Policy Research Institute 2010). Similar achievements were reported for the “Oportunidades” Program in Mexico (Rosenberg 2011).

In the Brazilian case, there has been an increase in the participation of individuals over 60 years of age in the national population from 4 % in 1940 to 8.6 % in 2000 and there are projections that they will reach 15 % in 2020. Moreover, the “very old” segment of the population (over 80) also increased dramatically in the past 40 years indicating that even the aged population is becoming older (Wong and Carvalho 2006). At the same time, some infectious diseases are still prevalent and endemic areas are part of the scenario. Therefore, there is still an increasing population of aged

people in Brazil living in remaining endemic areas for chronic infectious diseases such as schistosomiais, leishmaniasis, Chagas disease and malaria.

If we take schistome infection as an example, it will become clear the differences on the consequences of immunosenescence among geographically distinct populations. Usually, partial immunity to infection is acquired by adults between 25 and 49 years of age, with a further decline in infection seen in the following decades. However, Webster and coworkers (Webster et al. 1998) and our group in Brazil (Gazzinelli et al. 2001; Bethony et al. 2002) have reported that another rise in infection intensity occurs among the elderly, suggesting a loss of this partially acquired immunity by elderly people. Extensive water contact studies in the area showed that exposure measures in total body minutes were not statistically different among age groups nor they are different among individuals with distinct levels of infection (Gazzinelli et al. 2001). In short, the rise in egg counts during the last decades is not due to a change in exposure to infection but rather to some innate factor. However, the most interesting observation was that, in these areas, there are individuals in all age groups (including many over 70) that are tested negative for the presence of *S. mansoni* eggs (Bethony et al. 2002; Gazzinelli et al. 2001; Speziali et al. 2004) suggesting some individuals present robust mechanisms of protective immunity.

The concept of healthy aging has an immediate consequence for studies on aging and endemic diseases. Non-infected, negative aged individuals may be considered as an example of successful aging since they were able to develop compensatory mechanisms to cope with immune dysfunction and to generate protective responses against the constant threat of infection in their environment. Therefore, an important question for studies of aging and disease control in developing countries is which differences in the immunological profile of these negatively tested individuals can account for their resistance to either infection or re-infection.

Two mechanisms that we have investigated contribute to explain the differences between infection status in aged individuals in endemic areas for *S. mansoni* infection. First, egg negative aged individuals show a clear enhancement of innate immune responses that could participate in protective immunity and compensate for the aging-related deterioration in T-cell responses. This includes an increase in the frequency of IFN-gamma-producing CD16+ NK cells and also an increase in the frequency of CD56+ NK cells, dendritic cells and monocytes expressing TLR1 (Speziali et al. 2004; Comin et al. 2007). Second, infected aged individuals have chronically induced regulatory mechanisms that are more vigorous than negative individuals at the same age groups. Frequencies of three populations of regulatory T cells were higher in these infected individuals: CD4+IL-10+, CD4+CD25+Foxp3+ and CD4+LAP+ T cells (Comin et al. 2008). Although the action of these regulatory cells has probably beneficial effects on controlling disease-associated inflammatory damage, they may also hinder the development of protective immune responses. These preliminary observations suggest that, for aged individuals facing constant infection threat, inflammaging can be less deleterious than for elderly populations who inhabit clear environments.

Another conclusion from this novel perspective explored here for the healthy aging concept is that comparison between populations as distinct as Europeans and

Latin Americans, for instance, will have to take into account many factors related to genetic and epigenetic factors as well as environmental influences such as nutritional habits, cultural behaviors and autochthonous microbiota. Our hypothesis is that the chronic inflammatory status that accompanies the aging process (inflammaging) may result in distinct outcomes depending on the population examined. In times of demographical, economical and social changes in the whole world, further studies on the impact of the immunological changes during the aging process will have to take into account a diversity that has not been addressed by the previous studies on immunosenescence and human aging. It is likely that a much more universal concept of healthy aging will arise from such studies.

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Chapter 9
Mouse Models as Paradigms of Human Diseases

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Abstract This article invokes the distinction between early and late onset age-related disease to characterize the range of applicability of mice as models of human inflammatory disorders. We appeal to directionality theory, an analytical model of the evolutionary process, to propose that in early onset diseases, mice will represent reliable guides in studies of human disorders, whereas, in late onset diseases, due to species specific differences in the adaptive immune response, mice will be ineffective models.

We illustrate this tenet by appealing to studies of the etiology of Alzheimer's disease using murine models.

Keywords Alzheimer's disease · Animal models · Directionality theory · Entropic selection principle · Immune system evolution

9.1 Introduction

Mice and humans, as almost all species that belong to the genus Mammalia exploit similar genetic and epigenetic mechanisms to regulate processes of cellular growth and cellular differentiation. The two species share the same physiology, and at a certain level of organization, they show similar responses to various endogenous and exogenous perturbations. This similarity in physiology, and structure and response to certain pathogens, have been the rationale behind the extensive use of murine models to elucidate the mechanism of human diseases, and to furnish

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3_9,

therapeutic strategies to ameliorate these disorders. The diseases which have been given considerable attention are various forms of cancer, (Anismimov et al. 2005; Rangarajan and Weinberg 2003), and neurological diseases (Ashe and Zahs 2010).

Studies of the etiology of these diseases have provided great insight into common mechanisms. Tumorigenesis in both mice and humans is a multi-step process. These steps are genetic alterations that drive the progressive transformation of normal human cells into malignant derivatives. In many cancers, the genetic alterations in mice and humans are similar. This similarity has been the rationale for therapeutic strategies for humans based on extrapolating from studies using murine models. There is now some consensus that these extrapolations have not always been valid, see (Seok et al. 2013).

A similar situation arises in the study of neurological diseases. Studies based on murine models have been the basis for the amyloid cascade hypothesis, a model for the origin of Alzheimer's disease (AD). The model contends that the primary cause of AD is a dysregulation in the production and degradation of beta amyloid. This peptide is a derivative of the amyloid precursor protein (APP). Dysregulation in the production of beta amyloid is attributed to the effects of missense mutations in the APP gene. According to the amyloid cascade hypothesis, a viable therapeutic strategy for AD should be based on inhibiting the beta amyloid toxicity or targeting metabolic pathways involved in beta amyloid production. However, although strategies have been relatively effective in studies using murine models, they are largely ineffective in human clinical trials. A critical appraisal of some of these clinical trials is described in Hardy (2009) and Palmer (2011).

Although mice share genes, organ systems and systemic physiology with humans, the two species are characterized by significant differences in body size, one of the critical determinants of metabolic organization and function. Humans are about 3.000 times larger than mice. These differences in size entail significant differences in metabolic rate, the rate at which an organism appropriates resources from the external environment and converts these resources into usable energy. Body size also has an impact on metabolic stability, the rate at which the concentration of substrates and enzymes in cellular metabolic networks return to their steady state values after perturbations. These differences in metabolic rate and metabolic stability will impose dissimilar constraints on the response of mice and humans to various pathogens.

However, the argument that underlies the use of mouse models in the study of human diseases have in large part minimized these differences. The logic revolves around the following tenet: since the clinical and histological expressions of pathogenesis in mice and humans are similar, the diseases that they characterize must have the same etiology and consequently should respond to the same kind of therapeutic strategies.

This review will attempt to delineate the conditions under which mouse models are effective for understanding human diseases by distinguishing between the two systems in terms of their response to pathogens.

Our analysis is based on the application of directionality theory, a mathematical model of evolutionary dynamics, to the study of the comparative physiology and life-history of mice and human populations, (Demetrius 2013). A central feature of our analysis is that species differences in physiology and life history are the adaptive response of organisms to the different ecological constraints they have experienced in their evolutionary history.

Mice are opportunistic species: they spend the greater part of their evolutionary history subject to resource conditions which undergo large variations in abundance. Humans, by contrast, are equilibrium species: they spend the greater part of their evolutionary history under conditions where resource is relatively constant in abundance. These ecological constraints will impose contrasting responses in the life-history traits of the two species, in particular significant variations in life span and the rate of aging (Demetrius 2006).

Interspecific variations in the rate of aging will necessarily induce divergences in the response of the immune system to chronic inflammation. Now, all age-related diseases have an important inflammatory component. However, similar clinical and histological expressions of disease pathogenesis in mice and humans may not have similar etiology. The significance of this observation was recently illustrated by Seok et al. (2013) with an extensive analysis of genomic responses in mouse models of human inflammatory diseases. The authors show that although acute inflammatory stresses derived from different causes may result in highly similar genomic responses in humans, the responses in corresponding mouse models correlate weakly with the human responses. The study by Seok et al. concludes that, as a general rule, mouse models are an unreliable protocol for the study of human inflammatory diseases.

We may now ask: do there exist *specific* forms of human inflammatory diseases whose development can be reliably inferred from a study of murine models?

We will address this problem by appealing to the demographic-epidemiological concept, *age of onset*, to distinguish between two forms of human inflammatory diseases.

The term "early onset" is used to characterize disorders which are the result of a mutation in the nuclear genome. Such diseases affect individuals before middle age and are typically rare in incidence. The late onset form is generally the result of mitochondrial dysregulation. Age, in these diseases is the primary risk factor, and the disease occurs with a high frequency, and a risk which increases exponentially with age.

The distinction between early and late onset forms of the disease is well illustrated in the case of the neurodegenerative disorder, Alzheimer's syndrome (AD). The early onset form of the disease is associated with mutations in the APP gene and the secretase genes, presenilin-1, presenilin-2 and γ-secretase. The age of onset lies between 40 and 60 years. The late onset form of the disease is sporadic, with an age of onset of 70 years.

The age-distribution of individuals with autosomal dominant AD reflects the pattern observed in other human diseases with a Mendelian inheritance. The distribution is bell-shaped and reflects the condition that the age of incidence of the disease can be considered a variable, which is the multiplicative product of many independent random variables. The epidemiology in the sporadic form of the disease is quite different. The incidence of the disease increases exponentially with age (Swerdlow and Khan 2004). These differences in distribution indicate that although the early onset and the late onset forms of the disease may have indistinguishable clinical and histopathological signatures, the diseases may have quite distinct etiologies.

The early onset form of AD can be considered a *genetic* disease. The biochemical and histopathological signatures of the disease are the result of mutations in a family

of genes involved in the production and processing of the amyloid precursor protein. Since the genetic and epigenetic regulatory mechanisms in mice and humans are similar, the pathogenic molecules generated by the disease will induce similar inflammatory reactions in the two species. Similar anti-inflammatory drugs should have equivalent therapeutic effects on mice and men.

These observations suggest the following general characterization of diseases whose origin is primarily genetic:

I. In early onset forms of human inflammatory diseases, genomic responses in mouse and human systems will be indistinguishable. Accordingly, murine model systems will be reliable guides in the study of pathophysiological mechanisms in humans.

   Now the primary risk factor in late onset forms of human diseases is age. Aging, at the molecular level, can be considered as driven by an increase in molecular disorder. The aging process occurs because the changed energy states of biomolecules renders then malfunctioning and thus diminishes the efficiency of the metabolic processes within cells and tissues. This decline in efficiency will result in a decrease in the rate at which cells appropriate energy from their microenvironment and convert this energy into DNA, RNA and proteins. This cumulative increase in molecular disorder and decrease in metabolic efficiency will ultimately result in a dysregulation of the metabolic machinery (Demetrius 2013).

   The age-related property of late-onset forms of human diseases entails that these disorders are induced by mitochondrial dysregulation, and hence can be considered as *metabolic* diseases. Due to differences in evolutionary history, the stability of the metabolic networks in mice and humans will be characterized by significant differences. Consequently, the pathogenic molecules generated by late onset diseases will in general induce quite distinct inflammatory reactions in mice and men. Consequently, anti-inflammatory drugs may have quite dissimilar effects on the two species. We can therefore infer the following general characterization of diseases whose originis primarily metabolic:

II. In late onset subtypes of human inflammatory diseases, genomic responses in mouse and human models will be dissimilar. Consequently, murine model systems will be unreliable guides to the study of pathophysiological mechanisms in humans.

   There now exist several reviews which analyze differences between mouse and human immunology and the relevance of these differences in the study of human diseases, Mestas and Hughes (2004). This article will review these studies and appeal to concepts such as metabolic stability in order to furnish an evolutionary rationale for the distinction between the responses of early and late onset forms to the study of human diseases as described in (I) and (II).

9.2 Innate and Adaptive Immune Responses

The immune response is a complex process involving numerous organs and systems. Several comprehensive books have been written on general immunology (Murphy 2011; Abbas et al. 2011), and a plethora of reviews can be retrieved on

specific aspects of immune system functioning. In this chapter, we will restrict our discussion to those general aspects of the immune system which relate to evolutionary immunology (see Malagoli and Ottaviani 2010). The immune response can be functionally divided into two components—the innate and the adaptive components.

9.2.1 Innate Immunity

The innate immunity is always active, with various baselines of activation during life (Franceschi et al. 2007). The generalized defenses of innate immune system include the skin and the mucosae as an external first line of defense. These tissues present on their surface antimicrobial peptides, lysozyme and other soluble factors, whose action tends to reduce the risk of the penetration of pathogens (McDade 2003). Inside the body, cells endowed with phagocytic activity, such as macrophages and granulocytes patrol the whole body engulfing and eliminating non-self material. Complex processes such as inflammation, involve soluble factors, cells and other tissue components and flank innate immune elements, giving a significant contribution to the final outcome of the immune response (Ottaviani et al. 2010). The immune system in general, and the innate component, in particular, are deeply interconnected with the neuro-endocrine system, an overlap that seems to have an ancient origin (Ottaviani and Franceschi 1997; Adamo 2012; Geenen 2012). The innate immune response may be seen as a permanent arsenal, ready to be directed against all those components that are not recognized as "self". Innate immune components represent the oldest part of the immune system. They are already present in the progenitors of present-day vertebrates and extremely efficient also in relatively simple animals such as snails (Ottaviani 2006), worms (Bogaerts et al. 2010) and insects (Lemaitre and Hoffmann 2007). This means that the very basic components of the innate immune system are expected to be conserved and this is true if we maintain the view at a broad level. Every organism, however, developed a very specific arsenal, that it is usually proposed to be related to the environmental constrictions and the pathogens present in the colonized environment (Montaño et al. 2011).

9.2.2 Adaptive Immune System

The adaptive (or acquired) immunity, represents a different branch of the vertebrate immune system. The competences related to adaptive immunity are acquired during the first period of life (in our species, infancy and childhood) and refined during the lifetime (see Chap. 6, present book). Acquired immunity is a strictly individual characteristic because it has a strongly conserved genetic basis that may be differently expressed on the basis of life history. Pathogens encountered during IS maturation generate specific clones of cells, which are able to specifically address the immune response. The cells responsible for the emergence of an acquired immune

response are primarily the lymphocytes, subdivided in T and B lymphocytes. The molecules associated with adaptive immunity are the immunoglobulins (Igs), molecules of different size and organization that may be specifically directed against one epitope specific of the targeted pathogen (Murphy 2011).

From this description it might appear that innate and adaptive immunity can also be described in terms of "aspecific" and "specific" immune response, respectively, but this is not the case. Innate immunity presents an important degree of specificity, thanks to pathogen associated molecular patterns (PAMPs), and it significantly contributes to the subsequent adaptive responses (Shalhoub et al. 2011). The adaptive immune response achieves its relevance in terms of its short reaction time on a second encounter with the pathogen. This means that once a potential pathogen is encountered for the second time, it is quickly and specifically attacked by the IS, that contemporaneously employs both the innate (as a constant basis) and the adaptive (as additional and pathogen-tailored element) components of the IS.

9.2.3 Differences Between Mouse and Human Immunology

The presence of a clearly distinguishable acquired immunity is typical of vertebrates (Brehélin and Roch 2008), but in birds and mammals the secondary response displays a major quickness and effectiveness (Zimmermann et al. 2010). Mammals are the most studied group in terms of immunology, and an abundant literature exists on how the findings concerning one mammalian model is applicable to all the extant mammals. Notwithstanding this, several differences have been documented between mice and men—the canonical examples. These differences are largely dependent on size differences of the two animals (Sovak 1984).

In terms of innate immunity, for instance, differences in the biology of natural killer (NK) cells have been observed (Trinchieri and Perussia 1984). While in humans the NK activity is considered to be steady throughout life, the NK activity in mice is maximum in the first part of life. Moreover, NK cells present specific regulatory mechanisms in primates that are absent in the other mammals (Patham et al. 2010). Similarly, macrophages in human lungs appear to be more effective and spontaneously active than those in rodents (Haley 2003). The evolution of fundamental mediators of the innate immune response, such as the antimicrobial peptides of the defensin family, seems to have evolved according to different evolutionary constraints (Semple et al. 2006).

Acquired immunity is based on the same types of molecules in humans and mice. However, the acquired immune components in humans and mice present larger and more important differences than in the innate immune component. The main differences concern the principal elements of adaptive immunity, i.e. the immunoglobulins (Igs) and their receptors. A resume of all the differences observed between mouse and man goes beyond the aim of the chapter, but some significant examples may be reported. The mucosal associated IgA present relevant differences between man and mouse in terms of molecular organization, distribution and origin (Gibbons and Spencer 2011). Also the receptors for the constant fragment of the Ig

of class G have different expression patterns and present high diversity in terms of binding affinities (Bruhns 2012).

The comparison of the differences existing between murine and human innate and adaptive components indicate that the differences in the innate component usually concerns the level of the activity of the molecules (e.g. antimicrobial peptides) or the cells involved in the innate response. However, the adaptive component, especially at a molecular level, appears to be more diversified, and not surprisingly highly divergent between man and mouse.

9.3 Human and Mouse Cell-Mediated and Humoral Immunity

Besides the distinction between innate and acquired immune response, there exist another critical parameter for classifying IS. This revolves around the distinction between cell-mediated and humoral immunity. This parametrization distinguishes the major players of the IS, namely cells and molecules, irrespective of their involvement in innate or adaptive immunity. The distinction takes into account the functions of the single components (*e.g.,* a cell type or a molecular mediator) and does not consider the dynamics of the response or if there was a previous encounter with a pathogen. This second perspective underscores the basic components of the immune system, by emphasizing one specific cell type or a class of molecular mediators.

In mammals, the cells involved in immunity are myeloid cells (granulocytes and monocytes), lymphocytes and immune-regulatory cell types such as dendritic cells (Reizis et al. 2011). Among these three large families of cells it is possible to further distinguish numerous sub-populations (Shaloub et al. 2011).

The number of leukocytes circulating in mouse and human blood are significantly different (Haley 2003). Mice are characterized by a majority of lymphocytes. These include Ig producing cells, whereas humans produce a majority of neutrophil granulocytes (Doeing et al. 2003). These cells do not produce Ig and are short lived, active especially against bacteria and fungi (Murphy 2011). However, both mouse and human IS work quite well and it is unclear if owning a lymphocyte-rich or a neutrophil-rich blood results in important differences, at least in terms of fitness (Mestas and Hughes 2004). The immune-regulatory dendritic cells appears to be well conserved between mouse and humans (Shortman and Liu 2002).

Quite distinct from the relative low number of cell types, the humoral components are extremely numerous and include soluble factors such as cytokines, growth factors, antimicrobial peptides and many other molecules that contribute in orchestrating and modulating the cell-mediated immune response, beside directly killing the invaders (Ueno et al. 2010a, b).

Within species that are relatively similar in terms of phylogeny and that share very similar genes and cell types such as mouse and human, important differences do exist among the molecules produced and secreted by similar cells. For instance,

human neutrophils can produce the antimicrobial peptides defensins, whereas mouse neutrophils do not (Ganz 2003). Relevant differences have been observed in terms of receptors between human and murine monocytes (Ingersoll et al. 2010) and some cell receptors may be present on mouse but not human macrophages, (Shalhoub et al. 2011). More than a decade ago, it was already observed that murine macrophages can produce relevant amounts of nitric oxide, whereas human macrophages do not seem to possess the necessary metabolic pathways (Schneeman and Schoeden 2002, 2007).

Many other differences exist in secreted mediators of humoral immunity, both in terms of sequence and in terms of function. As an example, the pivotal and cytokine-based axis interleukin (IL)-12-IL-21 is operative in human but not mouse. Conversely, IL-6 is a potent inducer of IL-21 in murine but not human CD4+T lymphocytes (Schmitt et al. 2009; Ueno et al. 2010b).

As it has been described before in Sect. 2.3 for Igs and their receptors, the molecular players of immunity may exert highly different functions in mouse and humans, although the cellular types appear to be conserved.

9.4 Human and Mouse Immune Components: Implications for Experimental Models

This brief account of mouse and human immunology indicate that mice and men share basically, a common organization of the IS. Both the murine and human ISs function to enhance the viability of the organism, and at a superficial level they function in a very similar way. However, a deeper and more detailed analysis shows that mouse and human ISs are quite different, especially in terms of specific molecular components, that include Igs, cell-secreted mediators and receptors (Scheeneman and Schoenen 2007). This observation accounts for the failure of numerous extrapolations from murine to human systems: The success or failure of the extrapolation depends on the nature of the disease, which we characterize as early or late onset.

A specific genetic alteration generating an early onset disease in humans would more probably present a similar outcome in men and mice, since the genetic basis of the immune response and their general organization are conserved. Genetic-based diseases have a very basic and highly conserved characteristic. This property confers on genetic-based and early onset diseases a heritable condition not observed in sporadic diseases of animal models. As a consequence, data derived from animals that present similar genetic patterns may be applicable in analyzing disease etiology in human subjects.

As far as late onset diseases are concerned, the molecular variability existing even within individuals of the same species may be too wide to allow an effective analysis of human pathologies based on mouse models. Generally, sporadic diseases have a multi-factorial origin, and involve processes that have an evolutionary history.

One further element of diversity between mice and men derives from the fact that mammals are metagenomic organisms that are endowed with their own gene

sets together with those of all of their associated microbes (Ley et al. 2008). The composition of an individual microbiome should always be taken into account when investigating the etiology of late onset diseases (see Chap. 7 of this Book). Even though mouse and human intestinal microbiomes present a broadly similar composition, the differences between murine and human microbiota are much greater than those between individuals of one single species (Gibson and Spencer 2011). If we also consider this component, the molecular basis potentially underpinning late onset diseases is further enlarged and may thus further justify the discrepancies observed between experimental data collected in mice, and their application to humans.

9.5 Differences Between Human and Murine Populations: Evolutionary Origins

The immunological differences between mice and humans have their origin in the evolutionary history of the two species. This history is defined in terms of a two step process of mutation and selection, and modulated by the ecological constraints experienced by the species over evolutionary time.

Darwin's model of this evolutionary process is based on three fundamental tenets (Levins and Lewontin 1985):

I. *Variation*: the individuals in a population differ in terms of their morphology, behavior and physiology.
II. *Heredity*: there exists positive correlation between the phenotypic attributes of parents and offspring.
III. *Selection*: the resources which are necessary for survivorship and reproduction vary in term of their abundance and diversity. Individuals differ in term of their capacity to appropriate resources from the external environment and to convert these resources into net-offspring production.

These three tenets entail that the composition of the population will alter as new variants are introduced in the population by mutation, and natural selection discriminates between the variant and the ancestral type in terms of their capacity to exploit the available resources.

Evolutionary theory distinguishes between microevolutionary and macroevolutionary processes. Micorevolution is characterized by mutation and natural selection. This process occurs on a demographic time scale. It explains the changes in genotypic and phenotypic composition which results as one population type replaces another within a given lineage.

Macroevolution is defined in terms of the dynamics of speciation and extinction. These processes occur on an ecological and geological time scale. The genotypic and phenotypic differences which characterize mice and human populations are the result of the macroevolutionary constraints which have regulated changes within the mammalian lineage.

9.5.1 The Entropic Selection Principle

The main thrust of Darwin's theory is the notion that microevolutionary and macroevolutionary processes can be analyzed in terms of a selection principle which describes the outcome of competition for resources between variant and incumbent type.

Directionality theory, a mathematical model of the Darwinian argument, has furnished a formal characterization of a selection principle with explanatory and predictive value (Demetrius 2013). According to directionality theory, the outcome of competition between variant and incumbent types in a given population is described by the demographic parameter, evolutionary entropy, a measure of the variability in the age at which individuals reproduce and die. A population composed of individuals whose reproductive activity is distributed over several stages of the life-cycle, has large entropy. Among mammals, humans are the canonical example of a high entropic species. Among plants, perennials are characterized similarly by a complex life history. A population whose reproductive activity is confined to a few age classes has low entropy. Annual plants are a typical example of a low entropic population.

The entropic selection principle asserts that the outcome of competition between competing types is contingent on the resources and regulated by entropy. The principle involves two main tenets:

i. When resources are constant in abundance, and diverse in composition, variants with high entropy will have a selective advantage and will increase in frequency.
ii. When resources vary in abundance, and is singular in composition, variants with low entropy will have a selective advantage and increase in frequency.

Evolutionary entropy characterizes the demographic stability of a population. The parameter is positively correlated with the morphometric variable body size and the demographic parameter, life span (Demetrius 2013).

Evolutionary entropy is also correlated with the physiological parameter metabolic stability. This quantity, as observed earlier, describes the capacity of the metabolic network of an organism to return to the steady state condition when subject to random endogenous and exogenous perturbation.

We can therefore exploit the entropic selection principle to contrast the metabolic stability of mice and humans, as follows:

1. *Mice: a low entropy species*. These organisms will be characterized by a weak metabolic stability. Populations of mice will be demographically unstable. Total population numbers will be highly sensitive to small variations in the individual life-history.
2. *Humans: a high entropy species*. These organisms will be characterized by a strong metabolic stability. Human populations are demographically stable. Total population numbers will be largely insensitive to endogenous and exogenous perturbations.

9.5.2 Implications for the Immune Response

The preceding characterization of mice and human populations in terms of evolutionary entropy, provide a new perspective on differences in the immune response of the two species.

The immune competence is largely regulated by the metabolic stability of the organism. This property represents the response of the organism to pathogens and other agents which may affect the viability of the organism. Evolutionary entropy and metabolic stability are population and individual parameters constrained by the ecological constraints that regulate the resource abundance and diversity.

Resources which are constant in abundance and diverse in composition will confer a selective advantage to long lived species with strong metabolic stability. Resources which are variable in abundance and singular in composition will lead to short lived species with weak metabolic stability.

The implications of these effects on immune competence can be illustrated by contrasting mice and human. Mice and men differ not only in their life span but also in the duration of their intrauterine life, approximately 20 days for mouse and 40 weeks for man (Holladay and Smiallowicz 2000). In addition the degree of immune competence at birth is different between the two species.

The anatomical plan of the development of immune components and the contribution of liver and bone marrow is similar in the formation of stem cells and B lymphocytes, respectively (Holladay and Smialowicz 2000). The thymus is the site colonized by the T lymphocytes precursors developed by the fetal liver, early during development. In the thymus, the T-lymphocytes will mature and become able to discriminate between self and non self antigens (see Chap. 6). However, the timing at which the events occur, is not highly regulated being notably slower in mice. More importantly, the final result of the development is different, and therefore mouse and human immune–related cells are diverse in perinatal period. For instance, the thymic colonization occurs more early in humans than in mouse. The acquisition of a full functionality by B lymphocytes, fundamental players of adaptive immunity, and Ig producer, is significantly earlier in humans than in mice. In mouse the T-independent production of Igs largely precedes the T-dependent antibody response. In general, all the basic steps of immune development occurs earlier in humans than in mouse. As a result, the immune competence of a new born baby, though significantly lower than that of an adult man, is higher than that of a new born mouse (Holladay and Smialowicz 2000).

Accordingly, as a consequence of differences in evolutionary entropy, and concomitantly in metabolic stability, the IS of mice and men will be characterized by a significant variability. This variability will occur in both the innate and the acquired response. The latter, however, in view of its dependence on ecological constraints will be described by a variability with a wider spectrum.

9.6 Concluding Remarks

Mice are the cornerstone of *in vivo* studies of the immune response. In many respect, these organisms reflect human immunology, a condition which argues for their applicability to studies of the etiology of human inflammatory diseases.

Strong evidence of this immunological relation between mice and human system is indicated by comparative studies of mice and human genomes. As described by Waterson et al. (2002), only 300 or so genes appear to be unique to one species or the other. This genetic similarity is exploited in various investigations which have used murine models to investigate a wide repertoire of problems in human biology.

In spite of these genetic similarities, there exist significant differences at the metabolic level. This is reflected in the different responses of mice and human to caloric restriction, for example, and in differences in their innate and adaptive immune response.

These differences have an evolutionary rationale. They reside in the different ecological constraints that the two species have experienced. Such differences impose constraints on the evolutionary entropy of the two species. Mice, organisms subject to environments with resources of variable abundance and singular composition have low entropy, and concomitantly, weak metabolic stability and a high vulnerability to the action of pathogens. Humans organisms subject to environments with resources which are constant in abundance and diverse in composition, have high entropy. This property entails a strong metabolic stability, and a strong resistance to the action of pathogens.

The genetic similarities which the genomic analysis reveals, and the metabolic differences which the evolutionary perspective implies, have important implications in the study of murine systems as models of human inflammatory diseases. This implication can be summarized by distinguishing between genetic inflammatory diseases, which are primarily early onset disorders, and metabolic inflammatory diseases, which are age-related, late onset disorders.

Our analysis indicates the following distinction:

a. In early onset disorders, mice constitute reliable models of human diseases, and can be invoked in developing therapeutic strategies.
b. In late onset disorders, mice constitute unreliable models of human diseases, and may provide unreliable guides to protective methods.

The large failure of neuroprotective strategies based on murine models in studies of the sporadic forms of Alzheimer's disease, is consistent with this general tenet which distinguishes between early onset pathologies, which are primarily genetic, and late onset pathologies which are primarily metabolic.

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D. Malagoli, E. Ottaviani (eds.), *Eco-immunology*, DOI 10.1007/978-94-017-8712-3,