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General introduction
Diversity in the immune system

Diversity is a hallmark of the vertebrate immune system. Lymphocyte repertoires with millions of different specificities [10] function in concert with a large diversity of cytokines, chemokines, and different types of antigen-presenting cells (APCs) to protect vertebrates against infections. Different pathogens are handled by qualitatively different immune responses, varying from cellular to humoral responses, and varying in e.g. immunoglobulin isotype and cytokine expression [106]. At the same time, unwanted immune responses against self peptides and innocuous antigens are typically avoided. Due to the polymorphism of major histocompatibility (MHC) molecules, involved in antigen presentation to the vertebrate immune system, different individuals in a population typically respond differently to identical antigens.

The invertebrate immune system is far more primitive than the vertebrate immune system and lacks a diverse lymphocyte repertoire. Nevertheless invertebrates do respond effectively to pathogens and make a self–nonself discrimination [69]. Many components of these primitive immune systems have been preserved, and continue to play a crucial role in the vertebrate immune system. Although the focus of this thesis is on diversity in the vertebrate immune system, it is useful to start with a brief description of invertebrate immune systems.

Invertebrate immunity

Immune responses have been observed in invertebrates even as primitive as sponges [94]. Colonial invertebrates prevent invasion by members of their own species by distinguishing self from nonself and eliminating nonself components [69]. Allorecognition in invertebrates is typically mediated by histocompatibility molecules [42]. These highly polymorphic determinants expressed on the surfaces of cells allow organisms to maintain their genetic integrity [61].

The effector mechanisms involved in graft rejection in sponges are relatively simple and mainly rely on barrier formation and cytotoxicity [69, 201]. Antiparasite responses in higher invertebrates have been shown to be mediated by more sophisticated effector mechanisms. For example lectins, agglutinins and lysozymes play an important role in the elimination of pathogenic microbes via opsonization and lysis [69, 123, 128, 200]. Another example is the Toll protein, inducing antifungal and antibacterial peptides upon infection of Drosophila [120]. Additionally, phagocyte-mediated and killer cell-mediated defence responses have been observed in almost all invertebrates [18, 173].

A key characteristic of invertebrate effector mechanisms is their broad reactivity against groups of pathogens by recognition of conserved pathogen structures [104, 123, 142]. In order to avoid self destruction, those structures need to be distinct from the molecu-
ular structures occurring in self molecules. Examples of conserved pathogen-specific structures recognized by the invertebrate immune system are lipopolysaccharides and peptideoylglycans, both commonly expressed by bacteria [123]. Despite the broad reactivity of invertebrate immune responses, transplantation experiments have revealed that a kind of immunological memory occurs. Sea urchins transplanted twice with the same allograft showed a higher and faster second response as compared to the first transplantation response. This memory appeared to be nonspecific, as third-party allografts were shown to be cleared with a similarly increased efficiency [200]. Invertebrate memory typically lasts of the order of weeks or months, which is rather short as compared to the, sometimes life-long, immunological memory in vertebrates [94, 200].

There is increasing evidence that the vertebrate innate immune system is a homologue of the invertebrate immune system [104, 105, 133, 141–144]. One of the most striking examples of invertebrate immune components that have been preserved by the vertebrate innate immune system is the human homologue of the Drosophila Toll protein, which induces activation of human naive T lymphocytes [144] upon recognition of certain microbial products [220].

The transition from invertebrates to vertebrates

The phylogenetic transition from the invertebrate to the vertebrate immune system is marked by the appearance of adaptive immunity [69, 129]. Large repertoires of T and B lymphocytes with unique receptors on their surfaces form a second line of defence against infections, on top of the more conserved innate line of defence. The diversity of the adaptive immune system exceeds the total number of genes in any individual by orders of magnitude. Lymphocyte diversity is brought about by a series of somatic diversification mechanisms. Genes coding for the V, D, and J segments of lymphocyte receptors are somatically rearranged [4, 95, 229], and imprecise joining of the gene segments, addition of nucleotides, and somatic hypermutation subsequently increase the diversity of lymphocytes [106]. V(D)J recombination is mediated by the recombination-activating genes RAG1 and RAG2, which are thought to have once been part of a transposable element that became inserted into a receptor gene soon after the divergence of jawless and jawed vertebrates [4, 95]. From then on all vertebrates obtained the capacity to produce diverse antibody repertoires [127]. In contrast, there is no evidence whatsoever for the presence of rearranging immunoglobulins in invertebrate species [128].

Compared to the invertebrate immune system, the adaptive immune system functions in a fundamentally different way. Since lymphocyte repertoires are at least partially randomly generated, the adaptive immune system is not a priori specialized to recognize pathogen-associated molecular patterns. Instead it can respond to a virtually infinite variety of antigens as they are presented to the immune system. The random generation of lymphocyte receptors implies the need for self tolerance processes, because the distinction between self and nonself can no longer be germline selected [57]. Lymphocytes
could turn aggressive against self molecules of the host, a hazard termed *Horror Autotoxicus* by Ehrlich at the beginning of the twentieth century [71]. In the 1950’s Burnet came up with a solution to this problem of autoreactivity — a milestone in immunological thinking. He proposed in his *Theory of Clonal Selection* [41] that all lymphocytes are somatically tested for responsiveness to self molecules. Clones with self-reactive receptors are clonally deleted. All other lymphocytes remain quiescent until they are triggered by a specific antigen, allowing them to proliferate and to attain a higher precursor frequency. In fact, clonal selection is a “Darwinian corollary” [42, 58] because lymphocytes are subject to the same laws of mutation and selection as the individuals of a species.

**Interactions between innate and adaptive immunity**

The current consensus is that the innate and the adaptive part of the vertebrate immune system function in close co-operation [106]. When a naive vertebrate is infected by a pathogen, the immediate response is a nonclonal, innate response. Meanwhile an adaptive response may be induced. Importantly, the innate immune system has a pivotal role in the activation of the adaptive immune system. Merely the recognition of an antigen by a lymphocyte is not sufficient to initiate an immune response, and has been shown to cause T cells to switch to a suppressed state known as T cell anergy [107]. To overcome this activation problem, it is common practice in immunological experiments to induce adaptive responses by coinjection of complete Freund’s adjuvant. This is a mixture of killed mycobacteria in oil, which was aptly described by Janeway [104] as “the immunologist’s dirty little secret.” Adjuvants are thought to trigger the innate immune system, which subsequently provides costimulatory signals required to activate the adaptive immune system [74, 122]. The need for such “secondary signals” for the activation of lymphocytes was originally proposed by Bretsch & Cohn [38].

There is increasing evidence that the innate immune system imposes its evolutionary knowledge on the lymphocyte system, instructing it to mount an appropriate type of response [74, 75, 85, 104, 140–142, 144]. Depending on the context of an antigen, e.g. its localization [234], the presence of conserved pathogen-specific structures [104, 140, 141, 149], and any tissue damage [135], the immune system decides whether to respond or not, and if so which type of response to mount. Janeway [105] suggested that the innate signals allow the vertebrate immune system to distinguish between infectious nonself and noninfectious self molecules. An illustrative example of the importance of the context of antigens in the induction of adaptive responses was given by Ohashi *et al.* [159] and Oldstone *et al.* [160]. When viral proteins were converted into self antigens by inserting their genes into the germline of mice, they failed to provoke autoimmunity. The adaptive immune system was not tolerized by the viral antigens, but refrained from responding because the antigens were presented in a non-inflammatory context. Only when the mice were subsequently infected with the live virus [159, 160], did the lymphocytes attack the viral proteins and induce autoimmunity.
Finally, the effector phase of adaptive responses bears similarities with invertebrate immune responses. Specific binding between antibody and antigen, for example, triggers the complement cascade and attracts phagocytic cells and killer cells. Similarly, specific antigen recognition of T cells can lead to the release of nonspecific cytotoxic molecules. Specificity in adaptive immunity thus results from an antigen-specific release of nonspecific effector mechanisms [106].

Why adaptive immunity?

Thanks to the close co-operation between innate and adaptive immunity, the vertebrate immune system combines the evolutionary wisdom of the innate immune system with the large diversity of the adaptive system. The need for innate signals in the induction of adaptive responses, however, would allow pathogens to evade the adaptive immune response by evading the innate response. The seeming flexibility rendered by the random generation of lymphocytes is thus hampered by their requirement for innate signals [105, 142].

One burning question therefore remains: if the adaptive immune system hinges upon the innate immune system, and if invertebrates can perfectly do without it, then why did the adaptive immune system evolve at all? A common argument is that adaptive immunity enables vertebrates to remember immunological responses and thereby to respond more promptly upon reinfection thanks to increased precursor frequencies of antigen-specific lymphocytes (reviewed in [179]). As mentioned above, however, memory responses also occur in invertebrates. Indeed, there is no intrinsic reason why increased reactivity upon reinfection requires highly diverse lymphocyte repertoires.

Cohn [55] proposed that the need for an adaptive immune system arose when long-lived vertebrate organisms started to explore different ecological niches, and hence came into contact with a wide variety of parasites. Commonly used arguments for the absence of adaptive immunity in invertebrates are (i) that invertebrates are morphologically less complex than vertebrates, (ii) that invertebrates are typically smaller and thus have fewer cells than vertebrates, and (iii) that invertebrates are \( r \)-selected, while vertebrates are \( K \)-selected (reviewed in [179]). There are many counter-examples, however, of long-lived invertebrates such as corals, which may live up to hundreds of years, and invertebrates that are larger than particular vertebrates, e.g. octopi are larger than mice [179]. A satisfactory explanation for the lack of adaptive immunity in invertebrates, and its evolution in vertebrates thus remains elusive.

In this thesis we study what the adaptive immune system essentially adds to the innate immune system. We hypothesise that adaptive immunity stores immunological decisions in specific lymphocytes. Lymphocytes that have been instructed whether to respond, and if so which type of immune response to mount, recall this instruction whenever they recognize their specific epitope. This is a form of “acquired pattern recognition,” allowing
antigens to be promptly classified and dealt with. Being fairly independent of costimula-
tory signals [59, 77], instructed lymphocytes help to respond appropriately to antigens
that re-appear in a context that differs from their original context, e.g. pathogens that
hide in other tissues, or latent pathogens that temporarily do not cause any tissue damage.
In addition, instructed lymphocytes help to respond appropriately and promptly against
antigens that mutate during the life-span of a vertebrate, and against whole classes of
correlated antigens of which the immune system has encountered only a few members.
Storage of appropriate responses thus provides a selection pressure for the evolution of
adaptive immunity in vertebrates.

**Polymorphism of MHC molecules**

In addition to the diversity of lymphocytes there is another source of diversity in the
vertebrate immune system, which is due to variability in antigen presentation. For a T
cell response to be induced, the proteins of a pathogen need to be degraded into pep-
tides which are subsequently bound by MHC molecules and presented on the surface of
APCs [235]. The resulting MHC–peptide complexes are recognized by T cell receptors.
MHC molecules come in two classes: MHC class I molecules present peptides to CD8+
cytotoxic T cells, whereas class II MHC molecules interact with CD4+ T helper cells.
It has been estimated that more than half of the binding energy of T cell receptors to
MHC–peptide complexes is directed at the MHC helices, while the remaining energy is
directed at the presented peptide [125]. The most variable regions of the T cell receptor,
i.e. the CDR3 regions, have most contact with the peptide while the more conserved
CDR1 and CDR2 regions mainly interact with the MHC [88].

Just like invertebrate histocompatibility molecules, MHC molecules in vertebrates are
highly polymorphic. Some MHC loci have been shown to express more than one hun-
dred different alleles [166, 223]. Due to this high MHC population diversity, immune
responses of different individuals against identical antigens are typically directed against
different subsets of the antigen peptides. The polymorphism of MHC molecules be-
comes apparent when vertebrate tissues are transplanted from one individual to another.
Typically those transplantations evoke strong immune responses, eventually leading to
rejection of the tissue graft.

Although both MHC molecules and T lymphocytes are known for their extreme degrees
of diversity, the underlying mechanisms are fundamentally different. Whereas lym-
phocytes owe their diversity to special somatic diversification processes [106], MHC
molecules have mutation rates similar to those of most other genes [164, 184]. An ex-
planation for the high degree of MHC polymorphism can not be sought in vertebrate
allograft rejections, as these are experimental artefacts and thus not naturally involved
in evolutionary selection [61]. One possibility is that the vertebrate MHC polymorphism
is a “relict” of the invertebrate histocompatibility polymorphism [43]. Alternatively, the
selection pressure for MHC diversity may be due to peptide presentation to the immune
system. The two most commonly held views are that MHC polymorphism is due to selection favouring MHC heterozygosity [68, 99–101, 212] or due to selection for hosts with rare MHC molecules [19, 27, 195, 202].

Regarding the role of MHC molecules in pathogen presentation to the immune system, the number of MHC genes expressed per individual is surprisingly small. Each human individual expresses maximally six different classical MHC class I genes, and twelve different MHC class II molecules [167]. One would expect evolution to favour the expression of many MHC genes per individual. A solution to this paradox has been sought in self–nonself discrimination. A widely accepted argument is that excessive expression of MHC molecules leads to depletion of the T cell repertoire during self tolerance induction [54, 62, 157, 164, 211, 222]. In this thesis we dispute this argument and show that a different facet of self–nonself discrimination may be involved: the avoidance of inappropriate immune responses against self antigens that fail to induce tolerance limits an individual’s MHC diversity.

**Maintenance of lymphocyte diversity**

The peripheral lymphocyte repertoire is under homeostatic control. Despite de novo production of lymphocytes in the bone marrow and the thymus, and proliferation of peripheral lymphocytes upon antigenic stimulation, the total number of peripheral lymphocytes remains at a steady state. The mechanisms behind immune homeostasis are not fully understood, but there is increasing evidence that competition between lymphocytes plays an important role [80, 82]. It has been argued, however, that whenever different clones compete for the same ligand, the clone with the highest affinity for the ligand is expected to outcompete all other clones [63]. In ecology this is known as the Principle of Competitive Exclusion [86]. Competition between lymphocytes thus jeopardizes the maintenance of a diverse lymphocyte repertoire [88]. Indeed it has been shown that if the self-renewing T cell repertoire is maintained by stimulation with MHC–peptide complexes, the repertoire of stimulating peptides needs to be as diverse as the T cell repertoire itself [65].

Competition between different clones can also occur during the immune response to an antigen. T cells recognizing the same epitope from an antigen appeared to compete for limited antigenic stimulation [45]. This competition was shown to be epitope specific, because these T cells did not interfere with T cells specific for other epitopes of the same antigen [45]. The authors propose that upon antigenic stimulation, T cells compete for space on the APCs and for specific antigen-presenting sites. In this thesis we derive different T cell proliferation functions including T cell competition, and apply them to study the nature of T cell competition during immune responses. Our analysis confirms that T cells compete for antigenic sites on APCs. If APCs were to present epitopes of a single specificity only, T cell competition would cause the immune response to become monoclonal. We therefore propose that it is the variety of epitopes presented by
different MHC molecules on the surfaces of APCs, that causes immune responses to be multiclonal (i.e. typically oligoclonal). If the adaptive immune system stores appropriate responses in lymphocyte clones, it is of vital importance that immune responses are directed at multiple epitopes. It allows the immune system to recognize similarity between antigens in terms of overlapping sets of epitopes, and hence to use previous memory clones for the induction of the appropriate types of immune response against correlated antigens.

This thesis

In this thesis a variety of mathematical and computer simulation models are applied to study diversity in the vertebrate immune system. Part one of this thesis addresses the evolutionary selection pressures underlying the diversity of lymphocytes and MHC molecules. Part two deals with the maintenance of lymphocyte diversity during immune responses.

In Chapters 2 and 3 the evolution of lymphocyte diversity is studied. Previous mathematical models have suggested that the diversity of the adaptive immune system directly reflects the number of self antigens for which the immune system is tolerant [62, 152, 228]. Chapter 2 shows that storage of appropriate effector mechanisms requires a more specific lymphocyte system than was concluded from these previous models. Lymphocytes need to be specific to avoid autoimmune responses against self antigens that fail to induce tolerance, and to avoid inappropriate, cross-reactive responses against foreign antigens. Repertoire diversity allows the immune system to reconcile specificity with reactivity, which is needed to react to many different antigens [30, 34]. Chapter 3 gives a simulation model of an adaptive immune system that somatically learns to mount the appropriate type of immune response against different antigens. The model shows how memory lymphocytes may contribute in subsequent immune responses by providing signals about the context of novel antigens. The benefits of such a somatically learning immune system outweigh the accompanying risks if (i) the immune repertoire is sufficiently specific and (ii) there is some correlation between the antigens that are encountered [31].

Chapters 4 and 5 address the diversity of MHC molecules. In Chapter 4 several mechanisms are investigated to explain why the number of different MHC molecules expressed per individual is much lower than the MHC diversity at the population level. Using a probabilistic model, we demonstrate that it is unlikely that this results from repertoire depletion by negative selection in the thymus (cf. [62, 106, 157, 164, 211, 222]). Instead two alternative explanations are proposed. First, it is shown that thanks to the degeneracy of MHC–peptide binding, increasing an individual’s MHC diversity beyond 10–20 molecules hardly increases the likelihood that antigens are presented. Second, we show that the avoidance of inappropriate immune responses, such as autoimmune responses to ignored self antigens, yields a selection pressure decreasing an individual’s MHC
diversity. Chapter 5 demonstrates that despite this limited individual MHC diversity, host–pathogen coevolution can account for a very large population diversity of MHC molecules. Modelling the evolution of hosts and pathogens by computer simulation, we show that a high MHC diversity is to be expected in host populations adapting to pathogens with short generation times [21].

In Chapters 6 and 7 we study competition between lymphocytes during immune responses. In Chapter 6 we derive a proliferation function involving competition for a limited resource, which is applied to T cell proliferation. The function that we derive is an extension of the standard Michaelis–Menten approximation for enzyme–substrate reactions, which is frequently applied in theoretical models of the immune system. We show that our new proliferation function is valid in a wider parameter range than the conventional Michaelis–Menten approximation. In Chapter 7 our new proliferation function is applied to an experimental study of the role of T cell competition during immune responses. We use an in vitro proliferation assay in which both the concentration of T cells and the antigen availability are varied. By fitting different mathematical T cell proliferation functions to the in vitro data, we find — in line with previous experimental data [45] — that upon stimulation with antigen, T cells compete for antigenic sites on APCs.

Chapter 8 provides an overall discussion on the evolution and maintenance of diversity in the immune system.