

# Diversity in the Immune System

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# Diversity in the Immune System

Diversiteit in het Immuunsysteem  
(met een samenvatting in het Nederlands)

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# Contents

<b>1</b>	<b>General introduction</b>	<b>1</b>
<b>2</b>	<b>How specific should immunological memory be?</b>	<b>11</b>
<b>3</b>	<b>Adaptive immunity as a specific storage system of immunological decisions</b>	<b>25</b>
<b>4</b>	<b>What limits the individual MHC diversity?</b>	<b>39</b>
<b>5</b>	<b>MHC polymorphism: a result of host–pathogen coevolution</b>	<b>51</b>
<b>6</b>	<b>Extending the quasi-steady state approximation by changing variables</b>	<b>65</b>
<b>7</b>	<b>Competition for antigenic sites during T cell proliferation: a mathematical interpretation of <i>in vitro</i> data</b>	<b>85</b>
<b>8</b>	<b>Summarizing discussion</b>	<b>103</b>
	<b>Bibliography</b>	<b>109</b>
	<b>Abbreviations</b>	<b>127</b>
	<b>Samenvatting</b>	<b>129</b>
	<b>Curriculum Vitae</b>	<b>131</b>
	<b>Publications</b>	<b>133</b>
	<b>Acknowledgements</b>	<b>135</b>



# 1

## 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 molec-



ular structures occurring in self molecules. Examples of conserved pathogen-specific structures recognized by the invertebrate immune system are lipopolysaccharides and peptidoglycans, 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 Auto-toxicus* 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 Bretscher & 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 costimulatory 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 peptides 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<sup>+</sup> cytotoxic T cells, whereas class II MHC molecules interact with CD4<sup>+</sup> 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 hundred 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 becomes 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 lymphocytes owe their diversity to special somatic diversification processes [106], MHC molecules have mutation rates similar to those of most other genes [164, 184]. An explanation 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.





# 2

## **How specific should immunological memory be?**

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## Abstract

Protection against infection hinges on a close interplay between the innate immune system and the adaptive immune system. Depending on the type and context of a pathogen, the innate system instructs the adaptive immune system to induce an appropriate immune response. Here, we hypothesise that the adaptive immune system stores these instructions by changing from a naive to an appropriate memory phenotype. In a secondary immune reaction, memory lymphocytes adhere to their instructed phenotype. Because cross-reactions with unrelated antigens can be detrimental, such a qualitative form of memory requires a sufficient degree of specificity of the adaptive immune system. For example, lymphocytes instructed to clear a particular pathogen may cause autoimmunity when cross-reacting with ignored self molecules. Alternatively, memory cells may induce an immune response of the wrong mode when cross-reacting with subsequent pathogens. To maximize the likelihood of responding to a wide variety of pathogens, it is also required that the immune system be sufficiently cross-reactive. By means of a probabilistic model, we show that these conflicting requirements are met optimally by a highly specific memory lymphocyte repertoire. This explains why the lymphocyte system that was built on a preserved functional innate immune system has such a high degree of specificity. Our analysis suggests that (i) memory lymphocytes should be more specific than naive lymphocytes, and (ii) species with small lymphocyte repertoires should be more vulnerable to both infection and autoimmune diseases.

## Introduction

There is increasing evidence that the vertebrate innate immune system is a homologue of the invertebrate nonclonal immune system and that its evolution preceded the development of the adaptive immune system [104, 105, 133, 141–144]. Interestingly, the innate immune system was preserved when the adaptive immune system evolved. Innate immunity forms an essential part of the vertebrate immune system by providing signals for the activation of the adaptive immune system [75, 140–142, 144]. A hallmark of immune responses is the “second signal” [38] delivered to the adaptive immune system by innate antigen-presenting cells (APCs) that express the membrane proteins B7.1 and B7.2. In the absence of such costimulatory signals from the innate system, T cells fail to become fully activated and instead become anergic [107]. The adaptive immune system is thus dependent on evolutionarily conserved signals. We adopt the view that the innate system imposes its evolutionary knowledge on the lymphocyte system instructing it to mount the *appropriate* response [75, 104, 140, 141].

This dependence raises an evolutionary problem. It is often argued that the adaptive immune system evolved to cope with rapidly coevolving pathogens. The clonal distribution of randomly rearranged lymphocyte receptors renders a high flexibility, enabling the adaptive immune system to adapt more quickly to coevolving pathogens than

the innate immune system can. However, if an adaptive immune response depends strictly on the innate immune system, then pathogenic evasion of an innate response implies evasion of an adaptive immune response (see also [105] and [142]). Viruses have indeed been shown to interfere with the innate immune system by producing proteins, e.g. soluble cytokine receptors or proteins that regulate antigen presentation [17, 137, 196, 198, 199, 205], that put the immune system on the wrong track. Rapidly coevolving pathogens thus cannot explain why the adaptive immune system has evolved its diversity. Here, we hypothesise that the specificity of the adaptive immune system is used to specifically store the instructions given by the innate immune system. Using a probabilistic model, we demonstrate that this task is best performed if memory lymphocytes are highly specific.

## Building a “world view”

We adopt the view that the innate immune system provides signals about the context of antigenic epitopes [23, 52, 53, 75, 104, 140–142, 149, 182]. Depending on (i) the organ where the epitope is detected [234], (ii) the presence of conserved pathogen-associated molecular patterns [104, 140], and perhaps (iii) tissue damage [135], the innate system signals whether the antigen should be attacked and if so, by which immune effector mechanisms. We conjecture that the evolutionary information provided by the innate system is stored in specific lymphocytes by their switch from their naive phenotype to a particular responsive mode or to a nonresponsive mode. Lymphocytes can thus use their specificity to build up a “world view,” to learn which epitopes are dangerous, which are harmless, and which immune response is most appropriate [191]. They should switch to a tolerant mode, e.g. to anergy, whenever the innate system provides a harmless context, so that lymphocytes specific for self peptides, food antigens, and the intestinal flora can be rendered tolerant [148]. Conversely, in a harmful context, lymphocytes should be instructed to mount an appropriate immune response and to enter the solid tissue [44, 148, 234]. All instructed lymphocytes, *i.e.* not only conventional memory cells but also for example anergic cells, thus carry information about the appropriate response for the epitopes they recognize. In our view, immunological memory should thus also be regarded as a *qualitative* memory of the *type* of immune response to be made. On top of this comes the conventional quantitative form of memory in terms of increased precursor frequencies.

There is good evidence that during a secondary encounter of the same epitope, lymphocytes recall their appropriate response [115, 159, 160] and no longer wait for instructions from the innate system. An example, that a qualitative memory may enable lymphocytes to skip over the innate instructions, is the memory for responsiveness *versus* nonresponsiveness in mice transgenic for a lymphocytic choriomeningitis viral (LCMV) protein [159]. In mice expressing the LCMV protein on their pancreatic  $\beta$ -cells, LCMV-specific T cells were neither tolerized nor activated by the LCMV protein. On infection with LCMV, however, the cells became stimulated and caused T-cell-mediated diabetes. Ap-

parently, once the LCMV-specific lymphocytes had seen LCMV in an infectious context, they were instructed to an aggressive response, which was subsequently remembered such that the LCMV protein on the pancreas was regarded as a harmful antigen. Such an LCMV-specific response could not be induced by LCMV infection in LCMV-transgenic mice that had been tolerized with LCMV peptides [115]. Thus, nonresponsiveness *versus* responsiveness is qualitatively remembered by the immune system.

Another example supporting the concept of a qualitative form of immunological memory is the immunity against vaccinia virus (VV). VV is one of many viruses that express proteins interfering with the innate immune system. It prevents its own presentation on MHC molecules of infected cells, blocks the complement cascade and several cytokines, and neutralizes chemokines in the local environment [199]. Tackling the immune system at its innate base, the virus typically prevents the induction of an immune response and thus manages to escape. Yet, vaccination against poxviruses has been extremely successful [199]. Apparently, once an adaptive immune response has been triggered, the host is insensitive to the viral immune evasive strategies. Our interpretation is that a qualitative memory identifies the VV epitopes as harmful, thereby circumventing the need for further innate instructions and enabling the host to prevent secondary VV infections.

An immune system with qualitative memory has obvious advantages. The complex decision whether and how to react to specific epitopes needs to be made only once. Memory lymphocytes can thus prevent tissue damage by pathogens on reinfection and on pathogen dissemination to other organs. There is, however, a drawback. Instructed lymphocytes, which are fairly independent of further innate instructions, run the risk of mounting inappropriate cross-reactive immune responses. For example, self-reactive lymphocytes that have escaped self tolerance induction may become stimulated by a pathogen and subsequently become aggressive towards self [12, 232]. Additionally, memory lymphocytes may cross-react in response to subsequent pathogens [73, 114, 193] and induce a memory response of the wrong mode, e.g. Th1 instead of Th2. The immune system should therefore be specific enough to avoid such cross-reactivity mistakes. On the other hand, the immune system should be sufficiently cross-reactive to ensure an immune response against any pathogen. Here we develop a model to calculate the optimal degree of specificity of lymphocytes to fulfill both requirements.

## Specificity of memory

To calculate the optimal specificity of lymphocytes, we will define the probability  $P_s$  of surviving infection by any specific pathogen and calculate for which degree of lymphocyte cross-reactivity this probability is maximal. Let the degree of cross-reactivity of lymphocytes be called  $p$ , *i.e.* each clonotype has a chance  $p$  to respond to a randomly selected epitope. In a naive animal,  $p$  corresponds to a conventional precursor frequency. Species having evolved highly specific clonotypes have a low  $p$  value, whereas those

with cross-reactive clonotypes have a high  $p$  value. For simplicity, the affinity of clonotypes is not taken into account. A clonotype either responds to an epitope, if its affinity is higher than a certain threshold affinity, or fails to respond.

### Avoiding autoimmunity

To avoid autoimmunity, clonotypes responding to self epitopes should be rendered tolerant, *i.e.* removed from the functional naive repertoire. Consider an animal with  $R_0$  different lymphocyte clones, and let  $f$  be the fraction of all self epitopes  $S$  that induce self tolerance. The functional repertoire after tolerance induction  $R$  consists of all clonotypes that do not respond to any of the  $fS$  tolerizing self epitopes. Suppose the animal is infected by a pathogen, which for simplicity is represented by a single antigenic epitope. The chance of mounting an immune response  $P_i$  is the chance that at least one clone in the functional repertoire  $R$  will be stimulated by the pathogen, *i.e.*

$$P_i = 1 - (1 - p)^R, \quad (1)$$

where the expected functional repertoire size is

$$R = R_0(1 - p)^{fS} \quad (2)$$

(see [62] and [30] for similar derivations).

### Complete self tolerance induction

First consider the simple case that all of the animal's self epitopes induce tolerance, *i.e.* consider  $f = 1$ . In Figure 1a, the probability  $P_i$  of making an immune response is plotted against the cross-reactivity parameter  $p$ . If the immune system is very specific, there is a large chance that none of the clones will recognize the pathogen. On the other hand, if lymphocytes are very cross-reactive, self tolerance induction impairs the immune system by reducing the functional naive repertoire. The maximum value of  $P_i$  (denoted by the arrow in Figure 1a) is attained for  $p \approx 1/(fS) = 1/S$ . The optimal specificity to mount immune responses to foreign antigens thus reflects the number of self epitopes that induce self tolerance. This result is identical to the conclusion drawn from previous models [62, 152, 228], namely that immune systems are diverse primarily because animals have large numbers of self antigens.

### Ignored self

Healthy animals, however, harbour potentially autoreactive lymphocytes that seem to be ignorant of their specific self ligands [50, 185] and may cause autoimmunity after stimulation [12, 159, 160, 232]. After infection by a pathogen, self tolerance is assured only

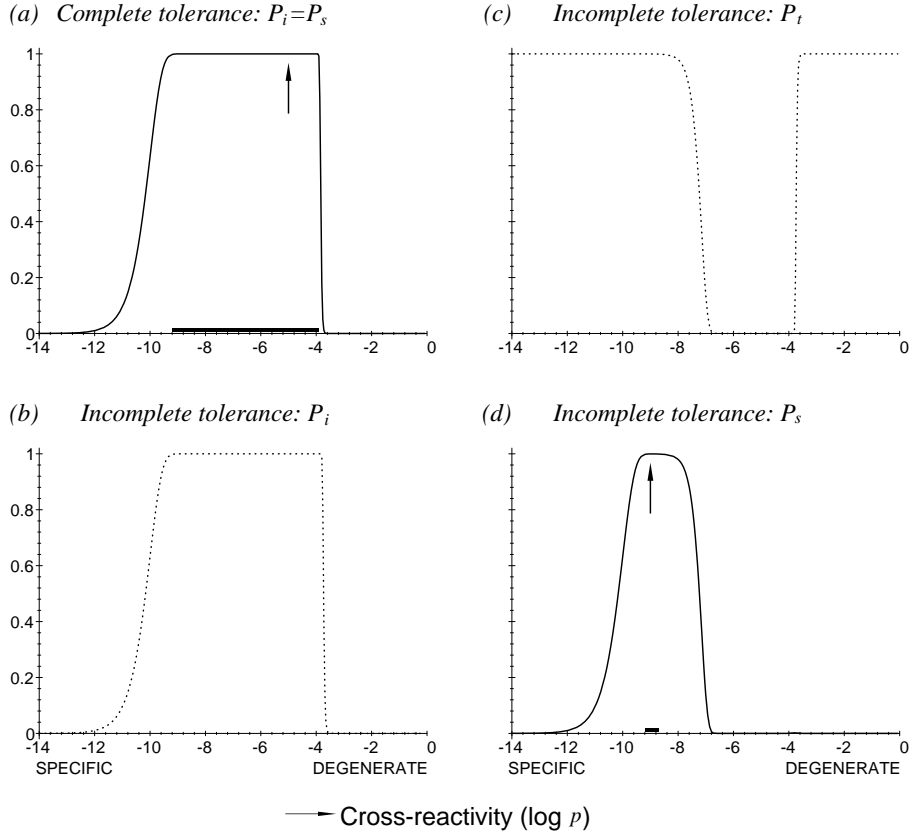


Figure 1. Avoiding autoimmunity. The chance of mounting an immune response  $P_i$  (a and b), the chance of remaining self tolerant  $P_t$  (c), and the chance of surviving a pathogenic attack  $P_s$  (d), defined by equations (1–5), plotted against the cross-reactivity  $p$  of lymphocytes. Specificity is simply the inverse of cross-reactivity. The arrows show that the optimal cross-reactivity in the case of complete self tolerance induction (a) is much larger than the optimal cross-reactivity when some self epitopes fail to induce tolerance (d). The black bars in a and d denote the specificity ranges for which the corresponding survival chances are close to the optimum, i.e. for which  $P_s^k > 0.9989$ , with  $k = 100$  different pathogens infecting a host (see text for further explanation). Parameters are  $S = 10^5$ ,  $R_0 = 10^{10}$ , and  $f = 1$  (a) or  $f = 0.8$  (b–d).

if none of the ignorant clonotypes is stimulated by cross-reactivities with this pathogen. Let  $\alpha$  denote the fraction of potentially autoreactive clones in the functional repertoire, i.e.  $\alpha$  is the fraction of clonotypes recognizing at least one ignored self epitope. Since only a fraction  $p$  of this subset of clones will be stimulated by the pathogen, the fraction of truly autoaggressive clones in the functional repertoire responding to a particular pathogenic epitope is  $p\alpha$ . The chance  $P_t$  of remaining self tolerant is the chance that none of the clonotypes in the functional naive repertoire falls in this autoaggressive cat-

egory. We are interested in the probability  $P_s$  that the animal will survive the pathogenic attack, *i.e.* in the probability that the animal will make an immune response *and* will remain tolerant to the ignored self, *i.e.*

$$P_s = P_t P_{(i|t)} = P_t - (1 - p)^R, \quad (3)$$

where  $P_{(i|t)}$  denotes the conditional probability of making an immune response given that the animal remains tolerant, and

$$P_t = (1 - p\alpha)^R, \quad (4)$$

and

$$\alpha = 1 - (1 - p)^{(1-f)S}. \quad (5)$$

Note that the intuitive interpretation of equation (3) is that the survival chance  $P_s$  is equal to the overall chance to stay tolerant minus the chance to stay tolerant by making no immune response at all.

The fraction of self epitopes that is ignored is unknown, but taking 20% as an example, the dashed curve in Figure 1c depicts the probability  $P_t$  that the system will remain tolerant to all ignored self epitopes when stimulated by a pathogen. This probability of tolerance  $P_t$  appears to be roughly inversely related to the probability of immunity  $P_i$ , denoted by the dashed curve in Figure 1b. This is because lymphocyte specificities that help epitope recognition, including self epitopes, will thwart self tolerance. The survival chance  $P_s$  is depicted in Figure 1d. The arrow in Figure 1d shows that the optimal lymphocyte specificity is much higher now than in the case of complete self tolerance induction. Prevention of autoimmunity to the ignored self apparently requires a high specificity (see also [30]).

If self tolerance induction is incomplete, the most important parameter determining the optimal specificity is the number of lymphocyte clones in the total repertoire  $R_0$ : the more lymphocytes are available, the more specific these lymphocytes should be (see Figure 2a). Highly specific lymphocytes reduce the chance of mounting autoimmune responses and thus increase the survival chance of the animal. Surprisingly, the number of self epitopes  $S$ , which largely determines the optimal specificity under complete tolerance induction, hardly affects the optimal specificity if self tolerance induction is incomplete. Neither does the fraction of ignored self epitopes  $(1 - f)$ , in that all curves for which  $f < 0.8$  are very similar to the  $f = 0.8$  curve.

In practice, selection for the optimal specificity might be hard to accomplish. Once a specificity has been selected for that gives sufficient protection against the typical total number of different pathogens infecting a host ( $k$ ), the driving force to evolve to an even better specificity vanishes. It might therefore be more informative to consider the range of specificities for which  $P_s^k$  is sufficiently large, say larger than 0.9. If an individual is exposed to about one hundred different pathogens on average, this range  $P_s^k > 0.9$  contains all specificities for which  $P_s > 0.9989$  (denoted by the black bars in Figures 1a

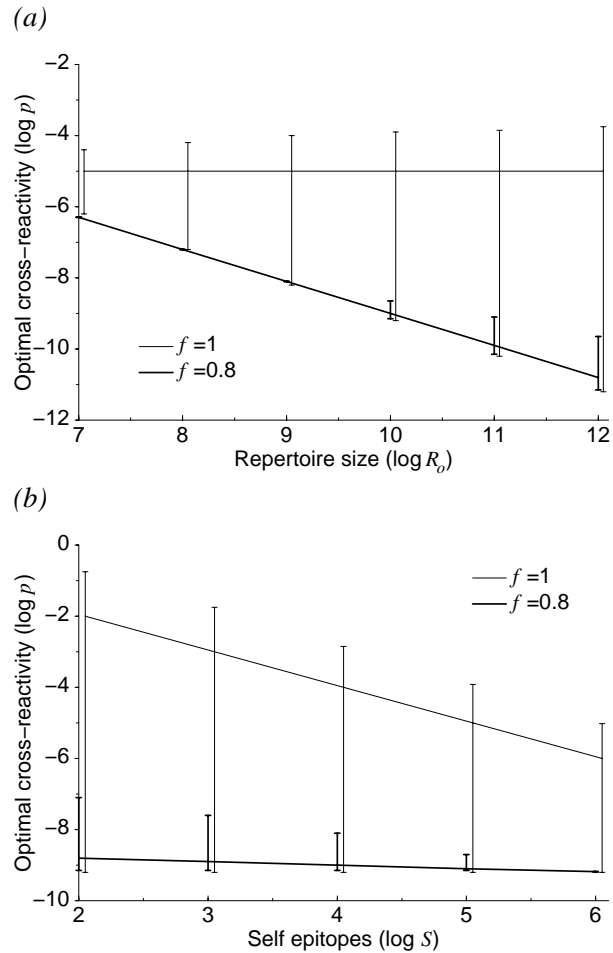


Figure 2. What determines the optimal specificity? The optimal cross-reactivity plotted against the size of the total lymphocyte repertoire  $R_0$  (a) or against the number of self epitopes  $S$  (b). If self tolerance induction is complete ( $f = 1$ ), the optimal cross-reactivity decreases as the number of self epitopes increases (b). The curves for which  $f = 0.8$  are typical for all cases of incomplete self tolerance induction ( $f < 1$ ). The optimal specificity in the case of incomplete tolerance induction is thus hardly dependent on the fraction of self epitopes that induces tolerance ( $f$ ). Results indicate that if self tolerance induction is incomplete, the optimal cross-reactivity depends mainly on the size of the lymphocyte repertoire (a) and is hardly dependent on the number of self epitopes (b).

and  $d$ , and the “error bars” in Figure 2). The specificity range for which  $P_s^k > 0.9$  in the case of complete self tolerance induction overlaps with that of incomplete self tolerance and is much wider. If self tolerance induction is complete, the optimal specificity level



is thus not defined as sharply as it is when some epitopes fail to induce self tolerance, and more particularly it is not as sharply defined as the  $1/S$  value suggested previously [62]. Summarizing, repertoires that run the risk of mounting autoimmune responses to ignored self epitopes should be orders of magnitude more specific than repertoires that need only to respond to many pathogens (*cf.* the recent paper by Mason [130]).

### Avoiding responses of an inappropriate mode

A second problem of cross-reactivity is that memory lymphocytes that have acquired a certain mode of immunity during a primary immune reaction may respond to subsequent pathogens [73, 114, 193] that require a different mode of response. Besides the widely accepted Th1 *versus* Th2 modes, many other modes of immunity may exist, varying in the type of lymphocytes, effector mechanisms, and cytokines involved [109, 110]. It has been demonstrated experimentally that the cytokine profile of a T cell response is determined by the cytokines present during lymphocyte activation (reviewed in [109] and [149]) and is epigenetically transmitted from mother to daughter lymphocyte [25, 26]. Thus, by secreting cytokines, cross-reactive memory cells may provide a wrong context for a primary immune response to be induced and can as a consequence impair immunity to subsequent pathogens.

The avoidance of such wrong mode responses is another driving force for the specificity of the adaptive immune system. Consider again an animal with a functional lymphocyte repertoire of  $R$  clonotypes (to exclude any effect of self tolerance induction, equation (2) is not yet substituted). The chance  $P_s(i)$  of surviving infection by the  $i^{\text{th}}$  pathogen, *i.e.* the chance of making an immune response without triggering any cross-reactive memory clonotypes, is now dependent on the fraction of memory clones in the repertoire  $m$ , and consequently on the number of previous infections  $(i - 1)$ . Only a fraction  $p$  of all memory lymphocytes will recognize the  $i^{\text{th}}$  pathogen, so that the fraction of clonotypes cross-reacting with the present and one previous infection is  $pm$ . The chance  $P_s$  to survive  $k$  different pathogens is the product of all survival chances from the first until the  $k^{\text{th}}$  pathogen, *i.e.*

$$P_s = \prod_{i=1}^k P_s(i) , \quad (6)$$

where, by analogy with equations (3–4),

$$P_s(i) = (1 - pm)^R - (1 - p)^R, \quad (7)$$

and

$$m = p(i - 1) . \quad (8)$$

Remember that any memory clone of an animal that has survived infection by  $(i - 1)$  different pathogens can, by our definition, be responsive to a single previous pathogen only.

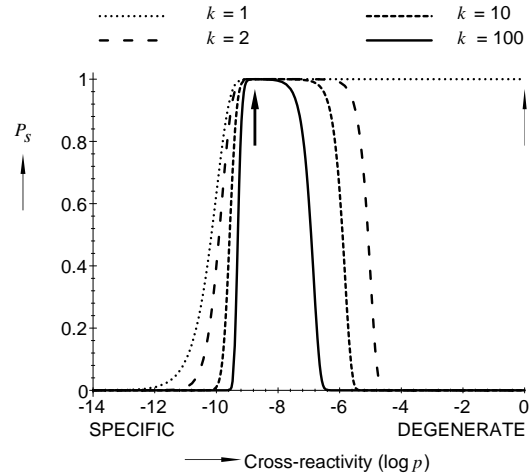


Figure 3. Avoiding responses of an inappropriate mode. The chance of surviving a single or multiple different pathogenic attacks, defined by equations (6–8), plotted against the cross-reactivity ( $p$ ) of lymphocytes. The curves denote the chances to survive infection by one, two, ten and one hundred pathogens, respectively. The optima of the latter three curves nearly coincide. The thick arrow denotes the optimum in the case of infection by one hundred pathogens. Results indicate that if an animal is exposed to multiple different pathogens, and thus runs the risk of mounting cross-reactive immune responses, clonotypes should be much more specific (see the thick arrow) than they should be if immunity against a single pathogen were the only demand (see the thin arrow). In the latter case, clonotypes should be maximally cross-reactive ( $p = 1$ ). Parameters are  $R = 10^{10}$ ,  $k = 1$  (dotted),  $k = 2$  (long dashed),  $k = 10$  (dashed), and  $k = 100$  (solid).

In Figure 3, the survival chance  $P_s$  is plotted for serial infection by various numbers of pathogens  $k$ . Figure 3 shows that the optimal specificity changes drastically from  $p = 1$  (i.e. 100% cross-reactivity), if the animal is exposed to only one pathogen, to a highly specific optimum, in the case of more pathogens. Immunological memory, and the accompanying risk of inducing inappropriate responses by cross-reactivity, thus forces the immune system to be specific. Again, it is the repertoire size  $R$ , and not the number of different pathogens  $k$ , that largely determines the optimal specificity (see Figure 3).

## Of mice and men

Because the optimal specificity to avoid cross-reactive immune responses is largely dependent on the size of the lymphocyte repertoire, our model predicts that the human and

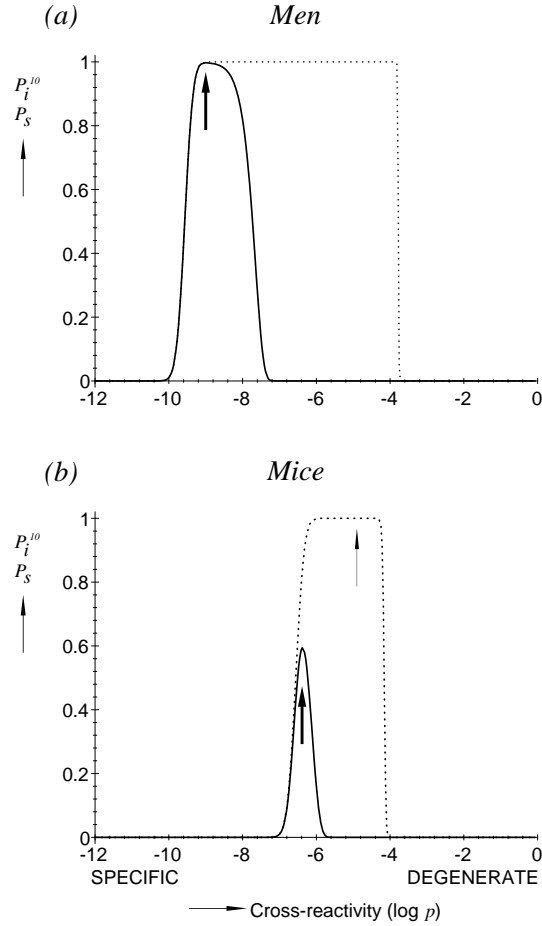


Figure 4. Of mice and men. Comparison of the optimal clonotype specificity for humans (a) and mice (b) if both types of inappropriate cross-reactive immune responses, *i.e.* autoimmunity towards the ignored self and mode selection failure caused by cross-reactive, old memories, can occur. The chance of surviving after infection by ten different pathogens ( $P_s$ , defined by equations (2), (5–6) and (8–9); solid), and the chance of mounting immune responses against those ten different pathogens ( $P_i^{10}$ , defined by equations (1–2); dotted), are plotted against the cross-reactivity  $p$  of lymphocytes. The optimal cross-reactivity of mouse and human lymphocytes are denoted by the thick arrows. Human lymphocytes should be orders of magnitude more specific than mouse lymphocytes. The thin arrow denotes the optimal specificity of mice clonotypes if resistance against many pathogens were the only demand. Parameters are  $S = 10^5$ ,  $f = 0.8$ ,  $k = 10$ , and  $R_0 = 10^{10}$  (for humans (a)) and  $R_0 = 10^7$  (for mice (b)).

the mouse lymphocyte systems may be quite different. To illustrate the predicted differences, the two models of the previous section are combined. The chance  $P_s(i)$  to survive

infection by the  $i^{\text{th}}$  pathogen is now the chance that none of the responding clonotypes is either a memory clone or a clone specific for an ignored self epitope, minus the chance that no immune response is made at all, *i.e.*

$$P_s(i) = (1 - p(m + \alpha))^R - (1 - p)^R, \quad (9)$$

where  $R$  is given by equation (2),  $\alpha$  by equation (5), and  $m$  by equation (8). The chance to survive  $k$  pathogens is still given by equation (6). In Figure 4, the chance of mounting ten immune responses ( $P_i^{10}$ , dashed curves), and the chance of surviving ( $P_s$ , solid curves) after serial exposure to ten different pathogens ( $k = 10$ ) are plotted. The total human lymphocyte repertoire is estimated to consist of  $10^{11}$ – $10^{12}$  T/B lymphocytes, whereas the mouse repertoire consists of approximately  $10^8$  lymphocytes [51, 116]. Taking an average clone size of ten lymphocytes per clone, we estimate the number of clonotypes in humans and mice to be  $10^{10}$  and  $10^7$ , respectively, *i.e.* a difference of three orders of magnitude. Figure 4 shows that at the optimum of the survival curve, human lymphocytes are orders of magnitude more specific than mouse lymphocytes. This is a new prediction. Previous models [62, 152, 228] have predicted that lymphocytes in mice and humans should be equally specific, *i.e.*  $p \approx 1/S$  (provided that mice and humans have similar numbers of self epitopes).

The need to avoid cross-reactivity with ignored self molecules and the avoidance of inappropriate cross-reactive memory responses are two independent driving forces for the specificity of lymphocytes. For the current parameter setting, the optimal lymphocyte specificity is mainly determined by the need to avoid autoimmune responses. For other parameter settings, *e.g.* for a lower number of self antigens  $S$  and a higher number of pathogens  $k$  with which an animal is typically infected, it may be the avoidance of inappropriate memory responses that determines the optimum of the survival curve.

In the optimum, the number of different clones responding to a pathogen is approximately the same for mice and humans. Thanks to the high specificity of human clones, humans should run a lower risk of mounting autoimmune responses than mice. The mouse immune system must make a concession: whereas its protection against infections could be just as good as that of humans (see the thin arrow in Figure 4*b*), the need to avoid inappropriate cross-reactive responses forces the mouse immune system to be more specific (see the thick arrow in Figure 4*b*). Thus, its resistance against infections is somewhat reduced. Summarizing, mice are predicted to have a smaller survival chance than humans because they suffer more from infections *and* from autoimmunity.

## Discussion

We have argued that the adaptive immune system specifically stores the instructions given by the innate immune system and that the specificity of lymphocytes is used largely for avoidance of inappropriate cross-reactive immune responses (see also [87]). It has been suggested previously that the diversity of the immune system reflects the

number of self epitopes that induce tolerance [62, 152, 228]. Here, we have shown that if there is any risk of inducing inappropriate cross-reactive immune responses, the immune system needs to be much more specific than had been derived from these previous models [62, 152, 228]. In particular, memory lymphocytes should not be triggered by cross-reactive stimulation by food or self antigens [216].

Intuitively, it is hard to see how responsiveness to foreign antigens and avoidance of inappropriate immune responses can be reconciled merely by selecting for a certain degree of lymphocyte specificity [130]. In our framework, however, there is an asymmetry between naive and memory clonotypes that allows this conflict to be solved. Inappropriate immune responses come from memory clonotypes only. In our model, naive clones do not run the risk of inducing an inappropriate immune response, because they either remain naive or are properly instructed to switch to the required phenotype. It is this asymmetry that allows for a high optimum of the survival curve at a high degree of lymphocyte specificity.

By considering the risk of cross-reactive autoimmune responses, we have implicitly calculated the optimal specificity of *memory* lymphocytes. Because naive lymphocytes do not run the risk of inducing inappropriate responses, it might be beneficial to have naive cells that are more cross-reactive than memory cells. Interestingly, naive B cells indeed appeared to react to a broader range of antigens than did memory B cells [207] (see also [126] and references therein). Because B cell hypermutation and affinity maturation occur largely after the primary immune response [106, 154], it is tempting to suggest that the function of B cell hypermutation is to induce highly *specific* memory B cells, on top of inducing a high *affinity* secondary response (see also [138] and [126], in which a more general form of specificity maturation was suggested). This idea is supported by the observation that beyond a certain avidity threshold there is no correlation between antibody avidity and protection against infection [13, 233]. Recent x-ray crystallographic studies uncovered a possible mechanism for specificity maturation: affinity-matured antibodies are more specific because they have a more rigid configuration than germline antibodies [227]. Selection for a high affinity thus seems to imply selection for a high specificity. It has been demonstrated that lymphocytes specific for self antigens are routinely generated during B cell somatic mutations [174]. In combination with the strong selective pressure on recognition of the original foreign antigen [14], specificity maturation may reduce the chance of releasing lymphocytes with cross-reactivity for self antigens into the periphery.

Throughout the calculations, the assumption was made that stimulation of a single clone is sufficient for a functional immune response. Obviously, this is a strong simplification. It is very likely that protection against infection and induction of autoimmunity require activation of multiple clones. We have chosen for maximal simplicity, however, because the qualitative results of the model do not depend on such complications. In their protection theory, Cohn & Langman [56] proposed that lymphocytes act in a concentration-dependent manner: to compensate for their larger lymph volume, large animals would require more lymphocytes of the same antigen specificity than small animals do. We can account for this argument in our model by considering the expected

repertoire size per unit volume. All calculations would remain the same, and our claim that immunological memory should be as specific as possible (per unit volume) remains true. It is only the predicted difference between large and small animals that disappears in the protection version of our model. The protection model need not be correct, however. Because of lymphocyte recirculation and homing to the sites of infections, large animals may indeed profit from their large lymphocyte repertoire. Even if this is only partly the case, our model correctly predicts a specificity and survival difference between mice and humans.

The high optimal specificities that we calculate seem to be at odds with recent measurements of precursor frequencies performed with MHC/peptide tetramers [78, 150] and with other estimates of lymphocyte cross-reactivity [130]. It should be stressed, however, that the optimal cross-reactivities calculated here reflect precursor frequencies in *naive* animals, which experimentally remain “soft numbers” [45, 150]. Naive precursor frequencies may be orders of magnitude lower than the precursor frequencies reported in MHC/peptide tetramer studies after immunization [78, 150]. Moreover, the precise quantitative results of our model depend on the specific choice of parameters and simplifications made (see also [30]). For example, we disregarded any safeguards that prevent cross-reactive cells from causing inappropriate immune responses [174, 234]. Additionally, there is no affinity in our model, whereas experimental estimates of precursor frequencies depend on the affinity cutoff of the specific assay that is used. Despite these quantitative complications, however, our results show that the need to avoid inappropriate immune responses imposes a strong selection pressure for the specificity of lymphocytes. Importantly, our model shows that the specificity constraints on lymphocytes are even stronger than was concluded previously [62, 152, 228].

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# 3

## **Adaptive immunity as a specific storage system of immunological decisions**

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## Abstract

During primary encounter of an antigen, the immune system has to decide which type of immune response is most appropriate. For instance, noncytopathic viral infections typically require a cellular response while elimination of most bacteria typically requires a humoral response. Such immunological decisions are based upon many factors such as signals from the innate immune system, and/or the local tissue environment. We hypothesise that the choice of the most appropriate type of immune response against each antigen is stored by the immune system in the form of differentiated clonotypes. Clonotypes that are triggered (or tolerized) by an antigen switch from a naive phenotype to a new, stable mode of responsiveness (or unresponsiveness), allowing the appropriate type of immune reaction to be regenerated upon restimulation of the clone. The adaptive immune system may contribute to the decision as to which type of immune response to mount, when novel antigens carry epitopes that the system has seen previously. This may go wrong, however, if differentiated cells coincidentally respond to new antigens. We develop a simulation model and a probabilistic model to investigate under which circumstances storing appropriate responses helps the immune system to rapidly make correct decisions. We find that lymphocytes need to be specific in order to avoid inappropriate, cross-reactive responses. Lymphocyte diversity is required to reconcile specificity with reactivity against many antigens. Increasing the diversity of the immune system does not hamper the positive contribution of memory lymphocytes in subsequent responses.

## Appropriate responses are stored by memory cells

Specific immunological memory is one of the most striking features of the vertebrate immune system. By inducing a cellular or humoral response to invading pathogens, the immune system is able to remove pathogens and to remember them specifically. Thus, vertebrates are able to respond faster and more efficiently upon reinfection. Adaptive immunity, and the accompanying ability of specific immunological memory, evolved at the transition from invertebrates to vertebrates, when gene rearrangements were employed to generate highly diverse lymphocyte repertoires [4, 69, 129]. Because lymphocyte receptors are at least partially randomly generated, the adaptive immune system requires self tolerance processes to avoid autoimmunity and mechanisms that allow naive lymphocytes to develop appropriate effector functions.

During primary antigenic encounter, there is a whole array of signals informing the immune system on the nature of the antigen that is being recognized [23, 75, 104, 105, 140–142, 182]. For example, the localization of the antigen [234], the presence of conserved bacterial peptides [104, 140, 141, 149], the type of tissue damage [135], and the cytokines and chemokines that are locally expressed [1, 158, 168], all influence the type of immune response that is induced. Based on these signals, which collectively form the



“context” of the antigen [52, 53], a complex decision is made as to whether to respond or not, and if so which effector mechanism to use. For efficient elimination, different pathogens require qualitatively different immune responses, varying from cellular to humoral responses, and varying in *e.g.* immunoglobulin isotype and cytokine expression [106, 209]. For example, antigen encounter in the gut will tend to induce IgA responses, gram-negative bacterial infections expressing LPS and causing tissue damage will generally trigger B cell responses, and recognition of viral RNA will typically induce a cytotoxic T cell response [106].

Over evolutionary time, certain antigenic contexts may have become correlated with the corresponding appropriate types of immune response. Alternatively, it has been proposed that the choice of immune response is determined somatically, by (success-driven) feedback mechanisms [190]. By whatever route the decision is made, we hypothesise that the adaptive immune system stores the appropriate modes of response against different antigens in differentiated lymphocytes. The immune system thereby somatically learns to associate the epitopes it has encountered with the appropriate modes of response against them.

## Memory cells influence subsequent responses

Once lymphocytes have been instructed as to which type of immune response to mount, they recall their appropriate mode of response when restimulated in subsequent infections [177, 209]. It has been shown that differentiated T helper cells recall their cytokine expression even in the presence of adverse costimulation (see [177] and references therein). Cytokine production is somatically imprinted in differentiated lymphocytes by chromatin remodelling and DNA demethylation. Instructed lymphocytes can thus epigenetically transfer their appropriate mode of response to their daughter cells [25, 26, 176].

Memory lymphocytes can greatly influence immune responses against subsequent infections. The ease with which they are triggered, even at very low antigen concentrations [5, 6, 40, 49, 170, 181, 204, 217], may explain why immune responses tend to be dominated by memory lymphocytes from previous infections, a phenomenon termed “original antigenic sin” [73, 114, 139]. It has for example been shown that the CD8<sup>+</sup> T cell response against influenza is dominated by memory cells that cross-react with previous influenza infections [92, 197]. Even for unrelated viruses such a bias to stimulation of previous memory clones has been observed [193].

Instructed lymphocytes can also direct the differentiation of other, naive lymphocytes [147]. CD4<sup>+</sup> T cells from transplantation-tolerant mice, for example, have been shown to render naive cells tolerant upon adoptive transfer. Since the so induced tolerant cells can in turn tolerize other naive lymphocytes this process was called “infectious transplantation tolerance” [98, 172, 225]. Later, it was demonstrated that infectious suppres-

sion can also take place between lymphocytes of different specificities. Anergic T cells, rendered anergic *via* T–T cell presentation of their antigen, appeared to actively suppress other T cell clonotypes, provided that both the anergic cells and the responder cells were confined to the same antigen-presenting cell [210]. Analogously, memory lymphocytes of a certain responsive mode may direct the differentiation of new, naive clonotypes, for example *via* cytokine secretion [168]. It has been proposed that T cells affect each other's differentiation *via* interactions with dendritic cells, which in turn promote the differentiation of responding T cells to different cytokine profiles [37, 178, 180, 208]. Cytokine-mediated T helper cell differentiation is characterized by a positive feedback: many cytokines promote their own expression by affecting the differentiation of particular T cell subsets [180]. Such a positive feedback has for example been observed for IL-2, IL-4, IFN- $\gamma$ , IL-10 [90], and TGF- $\beta$  [187]. Spreading of a responsive memory phenotype from one (self) epitope to another has also frequently been observed in autoimmune diseases [119, 218].

If cross-reacting memory clones happen to have the correct phenotype for a subsequent antigenic challenge, they are obviously advantageous to their host. Since memory cells are not confined to the lymphoid tissue and freely enter the solid tissue [44, 148, 234], they can respond anywhere and any time their specific epitope is encountered. Additionally, responses due to memory cells are typically more prompt than primary immune responses [5, 6, 40, 49, 59, 77, 170, 181, 204, 217], because memory cells are more sensitive to low antigen doses, have less stringent requirements for costimulation, and have already been instructed for the appropriate mode of response. Thus tissue damage by pathogens upon reinfection and upon pathogen dissemination to other organs can be prevented. Moreover, if pathogens mutate their antigenic structure, previous memory lymphocytes recognizing epitopes that have remained unaltered may direct the differentiation of new clonotypes recognizing altered epitopes of the pathogen.

Memory lymphocytes may also cause immunopathology, however. Being fairly independent of signals from the innate immune system and the local tissue environment, they run the risk of mounting inappropriate responses. Different antigens may possess overlapping epitopes and thereby trigger memory lymphocytes with inappropriate phenotypes. Moreover, self-reactive clonotypes that have escaped self tolerance induction may cause autoimmunity upon stimulation by external antigens [12, 159, 160, 232].

We have hypothesised in Chapter 2 that the immune system should be specific to minimize the risk of mounting inappropriate immune responses by cross-reactivity [30, 34]. Here a simulation model is developed to study under which circumstances immunological memory can help the induction of new, appropriate immune responses, while avoiding inappropriate cross-reactive responses. We find that both requirements are met whenever the lymphocyte system is sufficiently specific and diverse. Although this result may seem to be at odds with the high cross-reactivity of T cells proposed by Mason [130], a calculation in the Discussion shows that both views are perfectly compatible.

## A simulation model

To illustrate the basic principles of our hypothesis, we consider an immune system consisting of  $R_0$  clonotypes. Each clonotype has a certain mode, being either naive, tolerant, or responsive in a particular type of response. The modes are represented by integer numbers  $0, 1, 2, \dots, m$ , where 0 means naive, 1 means tolerant, and  $2, \dots, m$  identify the different types of responsive modes (such as Th1, Th2, IgA, IgE, etcetera). In the simulations presented here, there are ten different modes. When the immune system is challenged with an antigen we allow every epitope to be recognized by precisely one clonotype, which is selected randomly. Depending on the cross-reactivity of the system (here inversely related to its diversity), each clone may recognize multiple epitopes. Clonotypes specific for tolerance-inducing self epitopes are initialized in the tolerant mode; all other clonotypes are initially naive. At birth the system therefore consists of clonotypes with mode zero or one. Self tolerance induction need not be complete. In our simulations, a fraction  $f$  of the self-specific clonotypes is initialized in the tolerant mode; the other self-specific clonotypes remain ignorant of their respective self epitopes [50, 169, 185]. Such ignorant clonotypes may induce autoimmunity when they become triggered by pathogens [12, 159, 160, 232]. After birth the system is challenged with different antigens, each represented by  $e$  different (immuno-dominant) epitopes, and each requiring a certain mode of response. Both the appropriate mode of response to an antigen and the clonotypes recognizing its epitopes are selected randomly beforehand. Pathogens never kill their hosts, *i.e.* the simulations are continued even if an inappropriate response is induced.

Whenever epitopes of antigens in our simulations are recognized by previous memory clones, these memory clones determine what type of immune response is induced. The modes of response suggested by different memory clonotypes need not be identical, however. Any conflicts are resolved by treating each signal as a “vote” in the decision making process. The ultimate decision is the mode for which there is a majority count. In case there is a tie, the decision is chosen randomly from the largest votes. In the absence of cross-reacting memory lymphocytes we assume that the combination of the innate immune response, the context of the antigen, and possibly feedback mechanisms, ultimately leads to the appropriate type of immune response. This need not be unreasonable, because the innate immune system has learned about different kinds of pathogens and antigenic contexts over evolutionary time.

In our simulations, once the system has decided which type of response to make to a particular antigen, all naive clonotypes recognizing that antigen switch to the corresponding memory mode. Even if an inappropriate mode of response is triggered, naive lymphocytes switch (to the incorrect) mode. In accordance with experimental data [151, 176], memory clonotypes involved in a response to an antigen do not switch mode.

The performance of the model immune system is recorded by counting scores. In the default situation, in which a decision is made by the innate system and the clonotypes recognizing an antigen simply adopt the mode of the innate system, no score is given. All

Clone numbers:	0 1 2 3 4 5 6 7 8 9 10 11 12 13 ... $R_0$	
Initial modes:	0 0 0 0 0 0 1 0 0 0 0 0 1 0 ... 0	
	↓ ↓ ↓	
Antigen 1, mode 7:	7 0 0 7 0 0 1 7 0 0 0 0 1 0 ... 0	Zero score
	↓ ↓ ↓	
Antigen 2, mode 5:	7 5 0 7 5 0 1 7 0 0 0 5 1 0 ... 0	Zero score
	↓ ↓ ↓	
Antigen 3, mode 5:	7 5 5 7 5 0 1 7 0 0 0 5 1 0 ... 0	Positive score
	↓ ↓ ↓	
Antigen 4, mode 9:	7 5 5 7 5 5 1 7 0 0 5 5 1 0 ... 0	Negative score

Figure 1. A simple example of a simulation with  $e = 3$  different epitopes per antigen. After self tolerance induction most clonotypes are naive (*i.e.* mode 0), except clonotypes 6 and 12 which have been initialized in the tolerant mode (*i.e.* mode 1). The first antigen has to be rejected by an immune response of mode 7, and triggers clonotypes 0, 3, and 7. Since these three clonotypes are naive in the primary response, the decision as to which type of immune response to mount is made by the innate immune system. Thus, clonotypes 0, 3, and 7 become memory clones of mode 7, antigen 1 is rejected, and no score is obtained. Similarly, antigen 2 triggers three naive clonotypes, which subsequently switch to memory mode 5. Antigen 3 triggers two memory clones that overlap with antigen 2, *i.e.* clones 4 and 11, and triggers the naive clone 2. Because of the memory votes by clones 4 and 11, an immune response of mode 5 is triggered. This yields a positive score. Clone 2 correctly switches to mode 5. Antigen 4, requiring mode 9, coincidentally triggers a memory clone (2) which is in mode 5. Thus, an inappropriate immune response is induced, yielding a negative score. Naive clonotypes 5 and 10 incorrectly switch to mode 5.

cases in which previous memory clones establish the correct mode of response against an antigen (without being responsive to any self antigens) yield a positive score. The cases in which previous memory clones establish an incorrect mode of response against an antigen yield a negative score. This includes the cases in which the majority of the memory clonotypes involved is in the tolerant mode, and the adaptive system thus refrains from responding. We also score the number of autoimmune responses, which are induced when naive clonotypes that are ignorant of their self epitopes are triggered into one of the responsive modes, by pathogens cross-reacting with those self epitopes [12, 159, 160, 232]. An example of a small simulation is given in Figure 1.

Obviously, the adaptive immune system will only give a positive contribution to the decision making process if there are groups of structurally related antigens, *e.g.* coming from the same pathogen family or species, that require similar types of immune reactions. To account for such groups of antigens, a fraction  $P_m$  of all antigens in our simulations is a mutant of another antigen. Mutant and wild-type antigens always require identical modes of response and share half of their epitopes; the other epitopes are chosen randomly. All antigens are presented only once, *i.e.* we study a “worst case” sce-

nario, ignoring the conventional benefits of immunity obtained when the same antigen re-challenges the immune system.

### Somatic learning requires specificity

In Figure 2, the performance of immune systems that have been challenged with one thousand different antigens is plotted as a function of the immune system diversity  $R_0$ , and hence as a function of the specificity of clonotypes. Panels *a*, *b*, and *c* give the fraction of challenges that yield a positive score, a negative score, and an autoimmunity score, respectively. The different lines in the panels depict different degrees of correlation between the antigens, *i.e.*  $P_m = 0$  (solid),  $P_m = 0.1$  (dotted), and  $P_m = 0.2$  (dashed).

Figure 2*a* shows that memory clones help to make correct decisions whenever (i) there is some correlation between the antigens *and* (ii) the lymphocyte repertoire is sufficiently specific. At a very low repertoire diversity, hardly any positive score is obtained because most lymphocytes have been tolerized by self epitopes (see also [62]). At an intermediate repertoire diversity, the repertoire is no longer depleted during tolerance induction but the positive scores that are obtained are largely coincidental. Even if there is no correlation between the antigens (see the solid curve), these positive scores occur because of random cross-reactions. Above a diversity of  $R_0 = 10^5$  clonotypes, this randomness disappears and the positive scores hardly depend on the diversity of the immune system. Whatever the diversity of the system, a recurring epitope always triggers the same clonotype. Increasing the repertoire size  $R_0$ , and hence the specificity of the system, therefore does not impair the positive contribution of memory lymphocytes to the decision making during primary immune reactions.

At a low diversity, unrelated antigens expressing different epitopes and requiring different modes of immune response will tend to trigger the same clonotypes. Figure 2*b* illustrates that the adaptive immune system hence makes many mistakes. Previous memory clones recognizing epitopes of unrelated antigens tend to induce wrong types of immune response; clones that have previously been tolerized by self epitopes hinder the induction of immune responses to subsequent antigens. Figure 2*b* shows that such mistakes (i) disappear at a large repertoire diversity, and (ii) hardly depend on the correlation between the antigens.

Figure 2*c* demonstrates that at a very low diversity, autoimmunity hardly occurs. This is due to the large fraction of clonotypes that have been tolerized by self epitopes. At a somewhat higher diversity, many autoimmune responses are induced due to cross-reactions between foreign antigens and ignored self peptides. Such coincidental cross-reactions disappear if the immune repertoire is very diverse. Summarizing, Figure 2 illustrates that in immune systems that store the appropriate modes of response in differentiated lymphocytes, the benefits of immune memory outweigh the accompanying disadvantages whenever the immune repertoire is sufficiently specific.

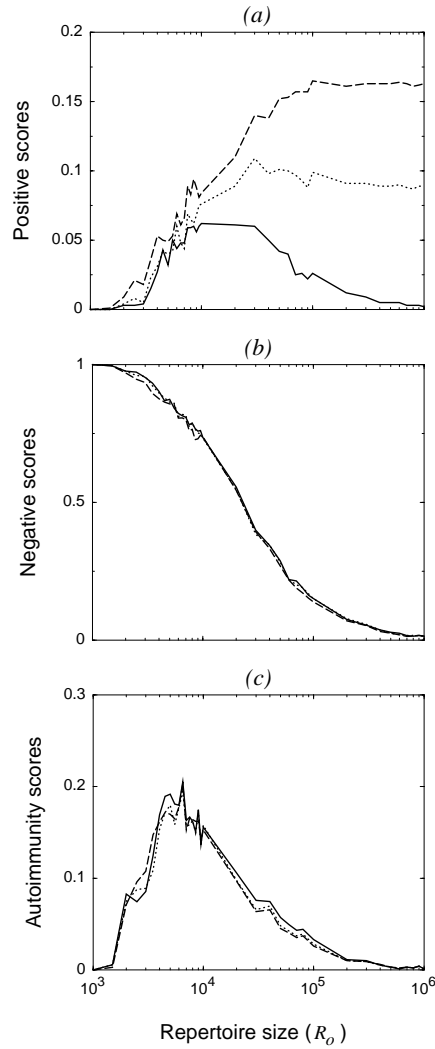


Figure 2. The performance of lymphocyte systems of different diversities ( $R_0$ ) challenged with one thousand different antigens. (a) The fraction of challenges that yield a positive score thanks to previous memory clones making correct decisions. (b) The fraction of challenges yielding a negative score due to inappropriate immune responses induced by previous memory clones or due to lack of responsiveness caused by cross-reactive tolerant clones. (c) The fraction of challenges leading to autoimmunity caused by ignorant, self-specific clones that are triggered by cross-reacting pathogens. The different curves denote different degrees of correlation between the antigens that are encountered:  $P_m = 0$  (uncorrelated antigens, solid curves),  $P_m = 0.1$  (dotted curves), and  $P_m = 0.2$  (dashed curves). Related antigens share 50% of their epitopes. There are  $e = 6$  different epitopes per antigen, a fraction  $f = 0.5$  of all  $S = 10^3$  self antigens induces tolerance, and there are ten different modes ( $m = 9$ ).

## A probabilistic model

In the simulation model described above, each epitope always triggers precisely one clonotype. Thus, the model’s immune repertoire never fails to recognize an antigen. It is more realistic to let clonotypes respond to epitopes with a certain probability  $p$ , so that any epitope triggers on average a fraction  $p$  of all clonotypes. In that case, specificity required to avoid inappropriate cross-reactive immune responses needs to be reconciled with sufficient cross-reactivity to allow immune responses against many different antigens. We employ a probabilistic model to show that this conflict can be solved if lymphocyte repertoires are sufficiently diverse (see also [30, 34]).

Let us consider an extreme scenario, by assuming no correlation between the antigens that are encountered and considering an infinitely large variety of types of immune responses. Thus there is no positive contribution of memory lymphocytes. Instead we focus on preventing inappropriate immune responses. Any situation in which an antigen triggers a clonotype recognizing a self epitope is considered to be wrong. We no longer distinguish between autoimmunity due to stimulation of naive self-specific lymphocytes, and absence of response due to tolerant lymphocytes instructing naive lymphocytes to adopt the tolerant phenotype. Just as in the simulation model, there are  $S$  different self epitopes and  $R_0$  clonotypes in the repertoire.

First consider the probability of mounting an appropriate immune response to an antigen that expresses  $e$  different epitopes. Having an infinite number of modes and no correlation between the antigens that are encountered, any responding memory clonotype is considered to cause an inappropriate immune response. Thus, an immune response will only be appropriate if all clonotypes responding to an antigen are neither self specific nor of the memory phenotype. In an animal with  $S$  different self epitopes that has previously encountered  $M$  different foreign epitopes, the probability  $v$  that a responding clonotype is naive (*i.e.* not responsive against any of the  $M$  previously encountered epitopes) and not specific for any self epitope is:

$$v \simeq (1 - p)^{S+M}, \quad (1)$$

where we write “approximately equal” because of possible overlaps between  $S$  and  $M$ . The probability that an antigen does not trigger any inappropriate immune response is the chance that each clonotype in the repertoire either fails to respond (with probability  $1 - p$ ), or responds and is naive and not specific for any self epitope (with probability  $pv$ ). This should hold for all of the  $e$  different epitopes of the antigen. Subtracting the probability that all clones fail to respond to the antigen gives the probability  $P_a$  of mounting an appropriate immune response:

$$P_a = (1 - p + pv)^{eR_0} - (1 - p)^{eR_0}. \quad (2)$$

## Repertoire diversity reconciles specificity with reactivity

In Figure 3 the probability of mounting an appropriate immune response is plotted as a function of the cross-reactivity parameter  $p$ , for immune systems that need to avoid cross-reactivity with  $S + M = 10^5$  different epitopes. A low value of the recognition probability  $p$  corresponds to a highly specific immune system; a high  $p$  value to a very cross-reactive immune system. From left to right, the four curves represent immune repertoires with  $R_0 = 10^{11}$ ,  $R_0 = 10^9$ ,  $R_0 = 10^7$ , and  $R_0 = 10^5$  clonotypes, respectively. Whatever the diversity of the immune system, there is an optimal level of cross-reactivity, above which many mistakes are made, and below which the immune repertoire frequently fails to recognize antigens [30, 34, 62].

The three left-hand curves all have a cross-reactivity region in which the induction of an appropriate immune response to an antigen is very likely, *i.e.*  $P_a \simeq 1$ . The width and height of this region increase with the diversity of the immune system  $R_0$ . At a low repertoire diversity ( $R_0 = 10^5$ ) many inappropriate immune responses are expected. Such inappropriate responses can be prevented in large immune repertoires by being

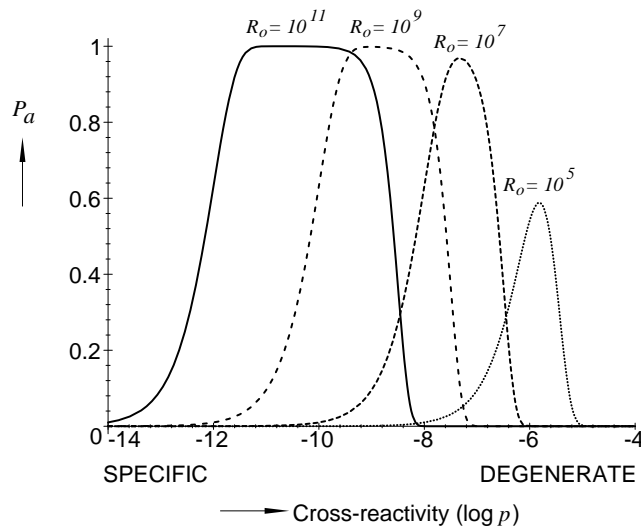


Figure 3. The probability  $P_a$  of mounting an appropriate immune response against an antigen with  $e = 10$  different epitopes, as a function of the cross-reactivity  $p$  of lymphocytes. Cross-reactivity with  $S + M = 10^5$  self epitopes and previously encountered epitopes needs to be avoided. If lymphocytes are very specific the immune system frequently fails to mount an immune response against an antigen; if lymphocytes are very cross-reactive many inappropriate immune responses are induced. Large immune repertoires can afford to be very specific, and thereby attain a larger maximum value of  $P_a$  than small immune repertoires.



sufficiently specific. Thus, high repertoire diversity reconciles specificity (to avoid inappropriate immune responses) with reactivity against many antigens.

### Inappropriate responses to novel antigens increase with age

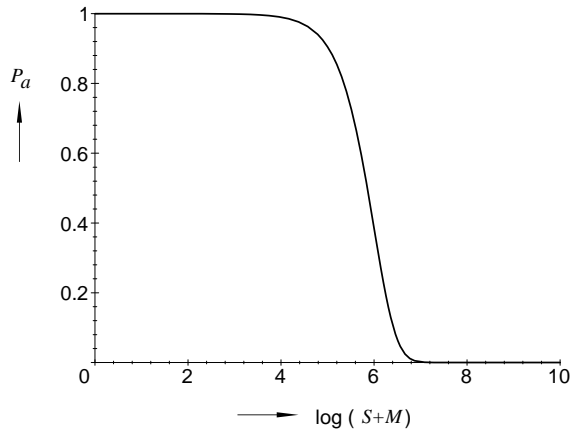


Figure 4. The probability  $P_a$  of mounting an appropriate immune response against a new antigen as a function of the (logarithm of the) number of epitopes  $S + M$  with which cross-reactivity should be avoided. Since any immune system will encounter more and more different epitopes with age, the horizontal axis also reflects the age of the immune system. Parameters are  $R_0 = 10^7$ ,  $e = 10$ , and  $p = 10^{-7}$ .

Since the adaptive immune system learns on a somatic time scale, adults tend to be better protected against infections than naive individuals. In the probabilistic model we have chosen to disregard any advantages conferred by memory lymphocytes. Although this choice is artificial, it reveals an interesting insight. Figure 4 shows that as the memory repertoire builds up with age, the chance to induce appropriate immune responses to novel antigens decreases. On the horizontal axis we have plotted the number of epitopes  $S + M$  with which cross-reactivity should be avoided. Since this number can only increase with age, it also reflects the age of the immune system. As the memory repertoire becomes more diverse, the chance increases that previous memory clones cross-react with new antigens and induce inappropriate immune responses. This is in good agreement with observations that childhood diseases such as measles and chickenpox typically cause more severe problems in adults than in children [28]. Although the naive repertoires of adults should still be sufficiently diverse to recognize any new antigen [10], we postulate that adult immune responses may be hampered by inappropriate effector mechanisms induced by previous memory clones.

## Discussion

We have studied the hypothesis that the adaptive immune system stores the immunological decisions made during primary immune responses in specific lymphocytes. Lymphocytes that have been instructed as to which type of immune response to mount recall this instruction whenever they recognize their specific epitope. This allows the immune system (i) to respond appropriately and promptly upon re-encounter of an antigen, even if some of its epitopes have mutated, and (ii) to respond appropriately to whole classes of correlated antigens, even if the immune system has been exposed to only one of their members. We conjecture that the qualitative property of a memory clone, *i.e.* its mode of response, is essential in immunological memory [181, 209], on top of the conventional increase in precursor frequency. An experimental comparison of naive and memory lymphocytes supports this idea: when equal numbers of naive and memory lymphocytes were transferred to Rag<sup>-/-</sup> mice, memory cells proliferated and performed their effector function much faster than their naive counterparts [84].

Conventionally, the immune system is thought to be diverse to guarantee an effective immune response to many different antigens (see for example [130]). In contrast, we argue that the high diversity of the adaptive immune system reflects the need to store appropriate modes of immunity against many different antigens in a very specific manner. If lymphocytes were to be degenerate, inappropriate cross-reactive immune responses would tend to be induced. According to our calculations, lymphocytes should be as specific as possible, within the constraints imposed by the size of the immune repertoire (see also [34]). Evolution would thus select for diverse, specific immune repertoires, with avoidance of inappropriate responses as the dominant selection pressure.

The diversity of the adaptive immune system has recently been estimated by Arstila *et al.* [10]. It was shown that the human naive T cell repertoire consists of *at least*  $2.4 \times 10^7$  different T cell specificities. The upper bound of the human naive T cell diversity was estimated to be  $10^8$  different clonotypes. Such a repertoire diversity would be perfectly functional according to our model. We have argued however, that the upper bound estimated by Arstila *et al.* [10] is probably several orders of magnitude too low [111].

Throughout this chapter we have adopted the premise that the innate immune system is capable of judging the infectivity of antigens [104]. Although not central to our argument, some problems remain. As pointed out by Bretscher [39], various non-pathogenic antigens, such as xenogeneic red blood cells and rhesus factor, induce strong immune responses even when administered without any adjuvant. Apparently, in the absence of innate signals, naive clones that become triggered nevertheless switch to some type of responsiveness, probably influenced by a context consisting of the local tissue environment only.

In our simulation model, we have allowed for an instructive role of memory clonotypes in the differentiation of other, naive clones. Upon encounter of their antigen, memory cells indeed recreate (at least part of) the cytokine context in which they themselves

were originally stimulated [180]. There is some evidence, however, that spreading of a memory phenotype from one clonotype to another may not always take place. The decreasing efficacy of repeated influenza vaccinations, for example, has been attributed to old memory clones preventing proper stimulation of naive clonotypes specific for novel epitopes in the vaccine [197]. Although we think the spreading of appropriate modes of responsiveness plays an important role in adaptive immunity, our results do not depend on this assumption. The fact that memory clones themselves have to respond appropriately is sufficient to explain why the adaptive immune system needs to be specific (simulation results not shown).

It has been proposed previously that clonotypes that have switched to a regulator phenotype due to self tolerance induction in the thymus may educate a “second wave” of clonotypes recognizing tissue-specific epitopes [147]. By analogy, in our model, tolerant clones may be helpful in preventing immune responses to antigens correlating with tolerance-inducing self molecules. Such an instructive role of tolerant clones could, however, be abused by pathogens. Pathogens could evade immune responses by the mere expression of proteins cross-reacting with self proteins of their hosts. Spreading of the tolerant phenotype to other clones recognizing truly foreign epitopes of the pathogen would hinder the induction of a protective response. It remains unclear whether tolerant clones specific for self epitopes can indeed obstruct the induction of an immune response against a pathogen. Induction of immune responsiveness by innate signals may overrule absence of responsiveness taught by tolerant clones. Additionally, the large population diversity of MHC molecules [21] may thwart immune evasive strategies based on self mimicry.

Since the theoretical number of different epitopes by far exceeds the size of any immune repertoire, clonotypes need to recognize multiple epitopes in order to ensure an immune response against any pathogen [130]. Mason [130] has estimated the total number of different immunogenic epitopes to be of the order of  $6 \times 10^{12}$ , and calculated that each clonotype in a repertoire of  $R_0 = 10^8$  clones should recognize at least  $3 \times 10^5$  different epitopes. Thus, it was concluded that T cells have to be “highly cross-reactive.” Our conclusion that lymphocytes should be highly specific (see also [30, 34]) seems to flatly oppose the conclusion drawn by Mason [130]. Both are fully consistent, however. In the same repertoire with  $R_0 = 10^8$  clonotypes and our “optimal” specificity of  $p = 10^{-7}$  (equation (2) with  $e = 1$ ), any epitope would trigger only ten clones, but any clone would recognize  $10^{-7} \times 6 \times 10^{12} = 6 \times 10^5$  different epitopes.

Summarizing, our models illustrate that specificity is a prerequisite of the adaptive immune system. Reliable storage of immunological decisions to many antigens in differentiated clones requires a highly diverse immune repertoire.



# 4

## **What limits the individual MHC diversity?**

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## Abstract

Although major histocompatibility (MHC) molecules are extremely polymorphic, the typical number of different MHC molecules expressed per individual is quite limited. A commonly used explanation for this limited individual MHC diversity is that excessive MHC expression would deplete the T cell repertoire during self tolerance induction. Re-examining a previously proposed model for the optimum number of MHC molecules, we here dispute this explanation. We show that depletion of the immune repertoire by expression of extra MHC molecules only occurs at an unrealistically high MHC diversity per individual. Two alternative explanations for the limited individual MHC diversity are proposed. Firstly, because MHC molecules bind their ligands with great degeneracy, expression of more than 10–20 different MHC molecules per individual hardly increases the chance to present and respond to antigens. Secondly, we show that a low individual MHC diversity helps to avoid autoimmune responses against self antigens that fail to induce tolerance.

## Introduction

Major histocompatibility (MHC) molecules, which are responsible for peptide presentation to the adaptive immune system, are known for their high degree of polymorphism. For some MHC loci more than one hundred different alleles have been identified [166, 223]. Nevertheless, the mutation rate of MHC molecules is comparable to that of most other genes [164, 184]. Since MHC genes are codominantly expressed, MHC heterozygous individuals can present a larger variety of peptides to the immune system than homozygous individuals can. MHC heterozygous individuals are therefore thought to have a selective advantage, being better protected against infections [68, 99–101, 212]. Indeed, in a study of patients infected with HIV-1, it has been shown that the degree of heterozygosity of MHC class I loci correlates positively with a delayed onset of AIDS [46].

In view of the role of MHC molecules in induction of immune responses, it is surprising that each individual expresses quite a limited number of different MHC genes. Just like favouring MHC heterozygosity, one would expect evolution to favour the expression of many MHC genes per individual. In reality, however, each human being expresses only three classical MHC class I genes (HLA A, B, and C), and three MHC class II gene pairs (coding for the  $\alpha$  and  $\beta$  chains of HLA DP, DQ, and DR). A heterozygous individual can therefore maximally express six different class I MHC molecules and twelve different class II MHC molecules (due to trans-association of the  $\alpha$  and  $\beta$  chains within HLA DP, DQ, and DR) [167]. It has previously been proposed that the optimal number of MHC molecules per individual is limited due to self tolerance induction in the thymus. During negative selection, clonotypes that recognize thymic MHC–peptide complexes with too high an affinity are deleted or rendered tolerant by other mechanisms

[155]. Excessive expression of MHC molecules might thus lead to depletion of the T cell repertoire [54, 62, 106, 157, 164, 211, 222].

Here we re-examine a previously proposed mathematical model for the optimal number of MHC molecules per individual [157]. We show that it is unlikely that the limited individual MHC diversity results from T cell deletion during self tolerance induction. Since MHC molecules positively select basically non-overlapping parts of the T cell repertoire (as has also been suggested by Bevan [24] and Fink & Bevan [76]), increasing an individual's MHC diversity tends to *increase* the functional T cell repertoire [70]. We propose two alternative explanations for the limited expression of MHC genes. Firstly, thanks to the degeneracy of MHC-peptide binding [83, 108, 132], increasing an individual's MHC expression beyond 10–20 different molecules hardly increases the likelihood that antigens are presented. Secondly, we show that the avoidance of autoimmune responses against ignored self antigens yields a selection pressure decreasing an individual's MHC diversity.

## Re-examining the optimal number of MHC molecules

Nowak *et al.* [157] developed a probabilistic model to test the verbal argument that T cell deletion due to self-tolerance induction limits the individual MHC diversity. The optimal number of different MHC molecules  $M$  per individual was calculated by maximizing the chance  $P_i$  to make an immune response against a single antigen (see also [62]). If antigens typically express  $e$  different (immuno-dominant) epitopes, this chance  $P_i$  is given by:

$$P_i = 1 - (1 - q + q(1 - p)^R)^{eM} . \quad (1)$$

Here,  $q$  is the chance that an MHC molecule presents a randomly chosen peptide,  $R$  is the size of the functional immune repertoire, *i.e.* the number of clones surviving both positive and negative selection, and  $p$  is the chance that a clonotype recognizes a random MHC-peptide complex. No immune response is induced if on all MHC molecules, all epitopes are either not presented (with chance  $1 - q$ ), or presented but not recognized by any of the  $R$  clonotypes in the functional repertoire (with chance  $q(1 - p)^R$ ). Equation (1) reflects the positive effect of expression of many different MHC molecules on the presentation of antigens.

The functional immune repertoire  $R$  in equation (1) is itself a function of  $M$ . Expression of many different MHC molecules reduces the functional T cell repertoire due to negative selection. On the other hand it enlarges the repertoire due to positive selection: only T cells that bind thymic MHC-peptide complexes with sufficient affinity enter the functional T cell repertoire [76, 224]. Nowak *et al.* [157] modelled the net influence of positive and negative selection on the functional repertoire as:

$$R = R_0(1 - (1 - h)^M)(1 - t)^M , \quad (2)$$

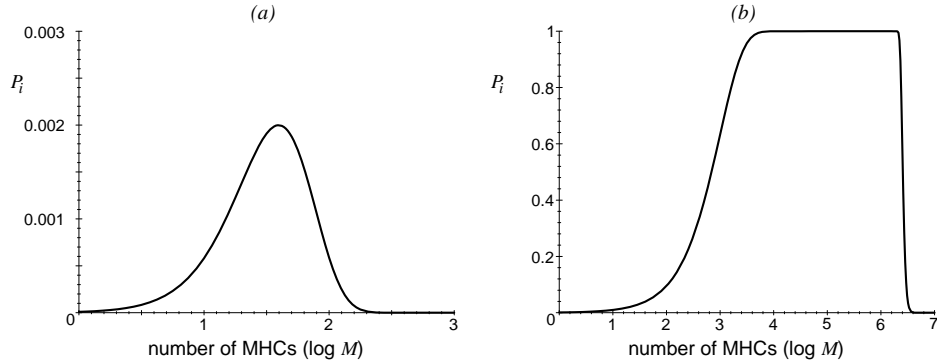


Figure 1. The chance  $P_i$  to mount an immune response as a function of the number of different MHC molecules per individual  $M$  for the parameter values proposed by Nowak *et al.* [157]. (a) The low optimum following from the model by Nowak *et al.* [157] (equations (1–2)) is due to too stringent negative selection, in which nonfunctional clones that fail to be positively selected on a particular MHC molecule can nevertheless be negatively selected on that MHC molecule. (b) Correction of this model (equations (3–5)) yields a much higher maximal  $P_i$  value with a wide maximum, coming down only at very high individual MHC diversities. Parameters are:  $e = 1$ ,  $q = 0.001$ ,  $p = 10^{-8}$ ,  $R_0 = 10^{10}$ . In the original model by Nowak *et al.* [157]  $h = 0.0001$  and  $t = 0.05$  (a). For one MHC molecule ( $M = 1$ ) these parameters are equivalent to  $h^* = 9.5 \times 10^{-5}$  and  $t^* = 5 \times 10^{-6}$  in the corrected model (b).

where  $h$  was defined as the chance that a clone is positively selected on a random MHC molecule,  $t$  as the chance that a positively selected clone is negatively selected on a random MHC molecule, and  $R_0$  as the total T cell repertoire before tolerance induction. In order to enter the functional T cell repertoire, clones need to be positively selected on *at least one* of the MHC molecules, but need to avoid negative selection on *all* of the MHC molecules of a host. T cells that fail to be positively selected on a particular MHC molecule, however, run at least a lower risk, and presumably no risk at all, to be negatively selected on that MHC molecule [7, 11, 186]. Thus, there is a problem with equation (2), which assumes that positive and negative selection on an MHC molecule are independent processes.

This problem becomes apparent when the chance  $P_i$  to mount an immune response against an antigen is plotted (see Figure 1a). At the optimum reported by Nowak *et al.* [157], having 40 different MHC molecules, the chance to respond to a single epitope is only  $P_i = 0.002$ . This low immunity chance results from too stringent negative selection. The immune repertoire is severely impaired, because T cells that fail to be positively selected on a particular MHC molecule can nevertheless be negatively selected on that MHC molecule. The negative selection term in equation (2) should instead only involve those MHC molecules that positively select a particular T cell. Having 40 different MHC molecules and a positive selection probability of  $h = 0.0001$ , this typically amounts to a single MHC molecule per clonotype.



Surprisingly, the solution to this problem is a reparameterization of equation (2). Let us first consider the fraction of thymocytes that is tolerized (with chance  $t^*$  per MHC molecule) by negative selection. Subsequently, we define the chance  $h^*$  that a T lymphocyte surviving negative selection is positively selected on a random MHC molecule. The functional repertoire is then given by:

$$R = R_0(1 - t^*)^M (1 - (1 - h^*)^M), \quad (3)$$

which is identical in form to equation (2), but has a different interpretation of  $t^*$  and  $h^*$ .

Similarly, equation (1) should be changed such that clonotypes only recognize peptides presented by those MHC molecules of the individual on which they were positively selected. Let us define  $R^*$  as the number of clonotypes from the functional repertoire that is expected to be positively selected by a particular MHC molecule of the individual, *i.e.*

$$R^* = h^* R_0 (1 - t^*)^M. \quad (4)$$

The chance  $P_i$  to mount an immune response against a random antigen then becomes:

$$P_i = 1 - (1 - q + q(1 - p^*)^{R^*})^{eM}, \quad (5)$$

where  $p^*$  is the probability that a clonotype in the functional repertoire recognizes a random peptide presented by an MHC molecule that the clone was positively selected on, *i.e.*  $p^* = p/h^*$ .

Figure 1b illustrates how these corrections influence the results by Nowak *et al.* [157]. If the parameter values of  $t$  and  $h$  used by Nowak *et al.* [157] are translated into the corresponding values for  $t^*$  and  $h^*$  in the corrected model,<sup>1</sup> there is a whole range of  $M$  values — varying from approximately 5000 to  $2 \times 10^6$  different MHC molecules per individual — for which immune responses are very likely to occur, *i.e.*  $P_i \simeq 1$ . For the parameter setting used by Nowak *et al.* [157], in which 0.01% of all T cells passes positive selection and 0.0005% of all T cells is negatively selected per MHC molecule, negative selection thus fails to explain why the number of different MHC molecules per individual is so limited.

## Better parameters for thymic selection

The parameter estimates used by Nowak *et al.* [157], however, are not in line with recent experimental data. It has been estimated that approximately 90% of all thymic T cells fails to be positively selected on any of the MHC molecules of a host [221]. At least 50%

<sup>1</sup>The parameter setting used by Nowak *et al.* [157] was  $h = 0.0001$  and  $t = 0.05$ , *i.e.* per MHC molecule 0.01% of all T cells passes positive selection and 0.0005% of all T cells is negatively selected. For one MHC molecule, this translates into  $h^* = h(1 - t) = 9.5 \times 10^{-5}$  and  $t^* = th = 5 \times 10^{-6}$  in the corrected model.

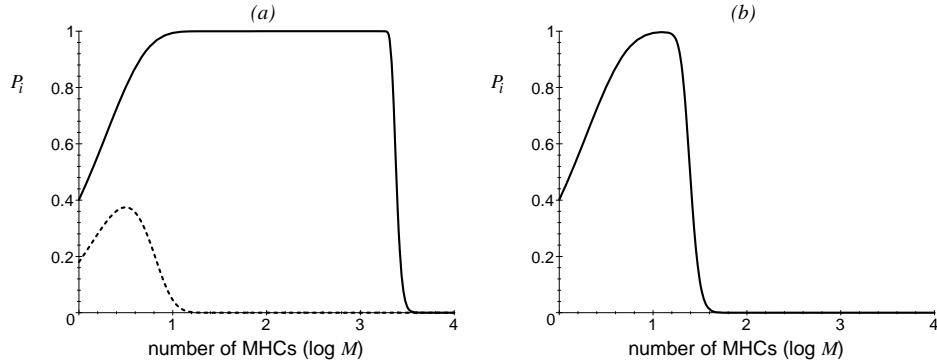


Figure 2. The chance  $P_i$  to mount an immune response as a function of the number of different MHC molecules per individual  $M$  for an experimentally based parameter setting. (a) The model by Nowak *et al.* [157] (equations (1–2), dashed curve) yields a much lower and more sharply defined optimum than the corrected model (equations (3–5), solid curve). Parameters are:  $q = 0.05$  [108],  $p = 10^{-8}$ ,  $R_0 = 10^{10}$  [157], and  $e = 10$  [62]. In the original model (dashed curve)  $h = 0.01$ , and  $t = 0.5$ , whereas in the corrected model (solid curve)  $h^* = 0.005$ , and  $t^* = 0.005$ . (b) The model by De Boer & Perelson [62] did not involve positive selection. To account for a thymic output of 5% (again with ten different MHC molecules), negative selection needs to functionally delete as much as 26% of the repertoire per MHC molecule. The curve (equations (3–5)) shows that in the absence of positive selection ( $h^* = 1$ ) and the presence of severe negative selection ( $t^* = 0.26$ ) one obtains a low optimal number of MHC molecules per individual and a high immunity chance  $P_i$ .

of all *positively selected* T cells has been shown to undergo negative selection in the thymus [221]. The remaining 5% of all thymic T cells ends up in the mature repertoire [60, 194, 224]. Since an individual has typically of the order of ten different MHC molecules, these estimates would translate into  $h^* = 0.005$  and  $t^* = 0.005$  per MHC molecule in the corrected model (equations (3–5)). In the original model by Nowak *et al.* [157] (equations (1–2)) it would amount to  $h = 0.01$  per MHC molecule. Thus, if there are ten different MHC molecules, positively selected lymphocytes are again typically selected on only one of them. To account for a 50% deletion of all positively selected cells due to negative selection, the chance to be negatively selected on such an MHC molecule should be  $t = 0.5$ .

Figure 2 illustrates that also for these experimentally based parameters, the original model [157] (dashed curve) yields a lower optimal number of MHC molecules  $M$  and a lower maximal immunity chance  $P_i$  than the corrected model (solid curve).<sup>2</sup> If T cells are only negatively selected on MHC molecules on which they are also positively selected, good protection is achieved for an MHC diversity between 10 and 2000 different molecules.

<sup>2</sup>For these parameters, the percentage of T cells surviving thymic selection by ten different MHC molecules is 5% in the corrected model and 0.01% in the original model [157].

Our model thus disputes the common idea that T cell deletion during negative selection explains why the individual MHC diversity is limited [54, 62, 106, 157, 164, 211, 222]. Since different MHC molecules select basically non-overlapping sets of T cell clones (*cf.* [24, 76]), addition of extra MHC molecules tends to enlarge the functional repertoire (see also [70]). Deletion of functional T cells by adding extra MHC molecules only occurs at unrealistically high individual MHC diversities. A low optimal number of MHC molecules can only be found if it is assumed that lymphocytes are very likely to be positively selected, as was done by De Boer & Perelson [62] (see Figure 2*b*). In their model, all deletion occurring in the thymus was due to negative selection. Obviously, in that case depletion of the T cell repertoire occurs even at low MHC diversities. A low optimal MHC number can, however, not be reconciled with the experimental finding that 90% of the T cell repertoire dies due to lack of positive selection [221]. Why then are there so few MHC molecules per individual? An interpretation suggested by Figure 2*a* is that, thanks to the degenerate binding of peptides to MHC molecules, a limited individual MHC diversity is simply sufficient to have a good chance to present and respond to antigens.

## Avoiding inappropriate responses

Another possibility is that the number of different MHC molecules per individual is limited to avoid the induction of inappropriate, cross-reactive immune responses. Inappropriate responses occur when different antigens requiring different modes of responsiveness trigger the same T cell clone [31, 34]. An example of an inappropriate response is when a self-specific clonotype that is ignorant of its self epitope is triggered by a cross-reacting foreign epitope and subsequently induces an autoimmune disease [12, 159, 160, 232]. The likelihood of such inappropriate immune responses increases with the number of epitopes that are presented to the immune system. Once there are sufficient MHC molecules to ensure presentation of antigens, having a greater diversity of MHC molecules may thus be detrimental.

To study this hypothesis we extend the model described above with the chance  $P_t$  to stay tolerant to all self peptides. This is expressed as the chance that during an immune response, on all of the  $M$  MHC molecules of a host, foreign epitopes are either not presented (with probability  $1 - q$ ), or presented but not recognized by any of the responding, ignorant self-specific clonotypes (with probability  $q(1 - p^* \alpha)^{R^*}$ ):

$$P_t = (1 - q + q(1 - p^* \alpha)^{R^*})^{eM} . \quad (6)$$

The probability  $\alpha$  that a clone from the functional repertoire is ignorant and self-specific<sup>3</sup>

<sup>3</sup>Note that equation (6) checks for each peptide–MHC complex from a foreign antigen whether the responding clones are autoreactive. Thus equation (6) gives an underestimation of  $P_t$  if clones recognize multiple peptide–MHC complexes coming from the same foreign antigen. In our parameter setting, this chance is negligible for  $M < 10^5$  since the probability that a particular clone recognizes an MHC–peptide complex during challenge with one antigen is  $M^* q e p^* < 5 \times 10^{-4}$ .

is given by:

$$\alpha = 1 - (1 - p^*)^{qS_i M^*}, \quad (7)$$

where  $S_i$  denotes the number of self epitopes that fail to induce self tolerance, and  $M^*$  denotes the expected number of MHC molecules that positively select one particular clone from the functional repertoire:

$$M^* = \frac{M h^*}{(1 - (1 - h^*)^M)}. \quad (8)$$

Note that the decrease in  $P_t$  with increasing  $M$  is due to (i) the increasing presentation of foreign epitopes, and (ii) the increasing fraction of ignorant, self-specific lymphocytes  $\alpha$ , due to the increasing number of peptide–MHC complexes formed by self antigens that fail to induce tolerance. The chance  $P_a$  to mount an appropriate response to an antigen is the chance  $P_t$  to stay tolerant minus the probability that all clones fail to respond:

$$P_a = P_t - (1 - P_i), \quad (9)$$

where  $P_i$  is given by equation (5).

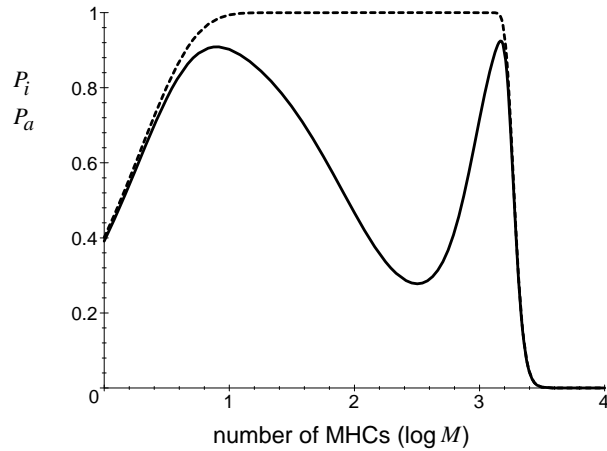


Figure 3. The chance  $P_a$  to mount an *appropriate* immune response, as defined by equations (3–9) (solid curve), as a function of the number of different MHC molecules per individual  $M$ . As a reference we have also plotted the  $P_i$  curve (dashed curve, equations (3–5)). Parameters are:  $q = 0.05$ ,  $p = 10^{-8}$ ,  $R_0 = 10^9$ ,  $e = 10$ ,  $h^* = 0.005$ ,  $t^* = 0.005$ , and  $S_i = 2 \times 10^4$ .

Figure 3 shows that involving the chance to mount an autoimmune response (solid curve) yields a sharply defined, low optimal MHC number, *i.e.* approximately eight MHC molecules per individual. Yet, the chance  $P_a$  to make an appropriate immune response in that optimum remains close to one. Apparently, the system can reconcile the need to respond to many antigens with the need to avoid cross-reactive, autoimmune responses, by selecting for a relatively low MHC diversity. At the left-hand top of the  $P_a$  curve,

adding MHC molecules hardly increases the chance  $P_i$  to mount an immune response against an antigen (see the dashed curve), while it significantly decreases the chance  $P_t$  to stay self tolerant. Interestingly, the curve in Figure 3 has a second peak at a high number of different MHC molecules per individual. At this right-hand peak, both self and foreign epitopes are presented as many different MHC–peptide complexes. The immune system then finds a balance between prevention of autoimmunity due to a severely depleted repertoire, and immunity against foreign antigens thanks to the formation of many different peptide–MHC complexes per epitope.<sup>4</sup> This scenario is extremely wasteful, since at the right-hand peak only 0.06% of the total T cell repertoire survives thymic selection.

If autoimmunity is less of a problem, the  $P_a$  curve no longer has two peaks. For example, if lymphocytes are highly specific (e.g.  $p = 10^{-9}$ ), the risk of autoimmunity by cross-reactions becomes negligible, and the  $P_a$  and  $P_i$  curves become virtually indistinguishable. Nevertheless, Figure 3 demonstrates that an increase in autoimmunity due to cross-reactions is a possible side-effect of expression of a large individual MHC diversity.

## Discussion

We have investigated why the number of different MHC molecules expressed per individual is so limited as compared to the large population diversity of MHC molecules. It is often quite loosely argued that individuals should not express too many different MHC molecules as this would lead to T cell repertoire depletion during self tolerance induction (see e.g. [54, 106, 164, 222]). Nowak *et al.* [157] translated this verbal argument into a mathematical model involving both positive and negative selection of T cells by MHC molecules in the thymus. We have re-examined this model [157], and have shown that it involved too stringent negative selection. T cells that failed to be positively selected on certain MHC molecules could nevertheless be negatively selected on those MHC molecules.

The present study shows that if T cells are only negatively selected on MHC molecules on which they could also be positively selected, adding MHC molecules tends to enlarge the functional T cell repertoire. Extra MHC molecules mainly tolerize lymphocytes that were not positively selected anyway in the absence of those MHC molecules. There is recent experimental support for a net enrichment of the T cell repertoire in MHC heterozygous hosts [70]. Our calculations show that depletion of the T cell repertoire by addition of extra MHC molecules is expected to occur only at unrealistically high MHC diversities. A low optimal number of MHC molecules can only be obtained if (nearly) all T cell deletion occurring in the thymus is due to negative selection, as was proposed by Matzinger [134] and modelled by De Boer & Perelson [62]. There is a

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<sup>4</sup>The position and height of the right-hand peak should be taken with care since our equations may become imprecise at very high values of  $M$ .

large body of data, however, showing that most thymic T cell deletion is due to lack of interaction with self MHC molecules [76, 221, 224]. We therefore conclude that T cell deletion during self tolerance induction fails to explain why the typical number of different MHC molecules per individual is so limited.

Unfortunately, direct estimates for the fraction of T cells that are positively and negatively selected *per MHC molecule* are not available. We have therefore used the total estimates of positive and negative selection [221] and the thymic output [60, 194, 224] to calculate the positive and negative selection parameters per MHC molecule in our model, assuming that an individual typically expresses ten different MHC molecules. This may seem a circular argument as we have thereby forced the equations to give realistic results at an individual diversity of ten MHC molecules. The bias works the other way around, however, because we find that T cell depletion *fails* to explain an optimal diversity of about ten different MHC molecules per individual.

Throughout our calculations, we have assumed that T cells can only recognize antigens presented on MHC molecules on which they could also be positively selected. The importance of T cell receptor binding to the MHC part of peptide–MHC complexes has been demonstrated experimentally. About 63% of the binding energy of a T cell receptor to a peptide–MHC complex has been shown to be directed at the MHC helices, while the other 37% is directed at the presented peptide [125]. Moreover, it has been demonstrated that T cell avidity for an MHC molecule contributes significantly to T cell specificity, and that it can even compensate for lack of avidity for the peptides presented by the MHC molecule [183].

Our model suggests that the limited MHC diversity per individual may be a direct consequence of degenerate MHC–peptide binding [83, 108, 132]. Once an individual expresses of the order of ten different MHC molecules, the selection pressure for more MHC diversity vanishes. This is in agreement with the fact that hardly any correlation has been found between particular MHC haplotypes and resistance against particular infectious diseases [171, 231]. Expression of 10–20 different MHC molecules per individual may be sufficient to present and respond to virtually any antigen. In contrast to the lack of correlations between MHC molecules and resistance against infectious diseases, strong correlations have been found between certain MHC haplotypes and susceptibility to autoimmune diseases [153, 231]. Such correlations are to be expected if autoimmunity is due to mimicry between foreign-peptide–MHC complexes and self-peptide–MHC complexes. We have extended our model with autoimmunity, by including ignorant self-specific clonotypes that can be triggered by foreign antigens. Our analysis demonstrates that avoidance of cross-reactive, autoimmune responses yields a selection pressure for a limited individual MHC diversity.

Despite the fact that different selection pressures may limit an individual’s MHC diversity, we will show in Chapter 5 that there will be selection for a large diversity of MHC molecules at the population level. A large population diversity of MHC molecules allows different individuals to respond differently to identical antigens, thereby giving protection against coevolving pathogens.

What limits the individual MHC diversity?

We would like to thank Can Keşmir and Joost Beltman for their significant contributions while discussing the concepts and equations of this chapter.





# 5

## **MHC polymorphism: a result of host–pathogen coevolution**

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## Introduction

There are ample examples of pathogens adapting towards evasion of immune responses. Viruses such as influenza rapidly alter their genetic make-up. Each year there appear to be sufficient susceptibles lacking memory lymphocytes from previous influenza infections to give rise to a new epidemic [36, 197]. During HIV infection, such alterations occur at an even faster rate, enabling the virus to repeatedly escape from the immune response within a single host [156]. Hosts, on the other hand, are selected for counteracting immune evasive strategies by pathogens. Since the generation time of hosts is typically much longer than that of pathogens, these host adaptations are expected to evolve much more slowly, however.

A well-known example commonly thought to reflect adaptation of hosts to pathogens is the polymorphism of major histocompatibility (MHC) molecules. MHC molecules play a key role in cellular immune responses. When a pathogen infects a host cell, the proteins of the pathogen are degraded intracellularly, and a subset of the resulting peptides is loaded onto MHC molecules, which are transported to the cell surface. Once presented on the surface of a cell in the groove of an MHC molecule, T lymphocytes can recognize the peptides of a pathogen and mount an immune response against them.

The population diversity of MHC molecules is extremely large: for some MHC loci, over one hundred different alleles have been identified [166, 223]. Nevertheless, the mutation rate of MHC genes does not differ from that of most other genes [164, 184]. Studies of nucleotide substitutions at MHC class I and class II loci have revealed that there is Darwinian selection for diversity at the peptide binding regions of MHC molecules. Within the MHC-peptide-binding regions, the rate of nonsynonymous substitutions is significantly higher than the rate of synonymous substitutions; in other regions of the MHC the reverse is true [99, 100, 164, 165]. Compared to the enormous population diversity of MHC molecules, their diversity within any one individual is quite limited (see Chapter 4). Humans express maximally six different MHC class I genes (HLA A, B and C), which are codominantly expressed on all nucleated body cells. Additionally, there are maximally twelve different MHC class II molecules (HLA DP, DQ and DR), which are expressed on specialized antigen-presenting cells [167]. The complete sequence of the human MHC has been unraveled recently [145]. Despite the high population diversity of MHC molecules, MHC genes appear to be extremely evolutionarily conserved. Allelic MHC lineages have persisted over long evolutionary time spans, often predating the divergence of present-day species [112, 113, 118, 136]. As a consequence, individual MHC alleles from a species tend to be more closely related to particular MHC alleles from other species, than to the majority of alleles occurring within the species [165].

Due to the high population diversity of MHC molecules, different individuals will typically mount an immune response against different subsets of the peptides of any particular pathogen. Pathogens that escape from presentation by the MHC molecules of a particular host, may thus not be able to escape from presentation in another host with different MHC molecules. MHC polymorphism may therefore seem a good strategy of

host populations to counteract escape mechanisms of pathogens. This is a group selection argument, however, which fails to explain how such a polymorphism could have evolved [27].

The mechanisms behind the selection for MHC polymorphism have been debated for over three decades. A commonly held view is that MHC polymorphism is due to selection favouring heterozygosity. Since different MHC molecules bind different peptides, MHC heterozygous hosts can present a greater variety of peptides, and hence defend themselves against a larger variety of pathogens compared to MHC homozygous individuals. This hypothesis is known as the theory of “overdominance” or “heterozygote advantage” [68, 99–101, 212]. A recent study of patients infected with HIV-1 supports this theory. It was shown that the degree of heterozygosity of MHC class I loci correlated with a delayed onset of AIDS. Individuals who were homozygous at one or more loci typically progressed more rapidly to AIDS [46].

It has been argued that selection for heterozygosity alone cannot explain the large MHC diversity observed in nature [165, 230]. Although there is general agreement upon the significance of overdominant selection, it has been proposed that additional selection pressures should be involved in the maintenance of the MHC polymorphism [165, 230]. A frequently studied additional mechanism is “frequency-dependent selection.” This theory states that evolution will favour pathogens that avoid presentation by the most common MHC molecules in the host population. Thus there will be a permanent selection force favouring hosts that carry rare, e.g. new, MHC molecules. Since hosts with rare MHC alleles have a higher fitness, the frequency of rare MHC alleles will increase, and common MHC alleles will become less frequent. The result is a dynamic equilibrium, maintaining a polymorphic population [19, 27, 195, 202].

Both selection for heterozygosity and frequency-dependent selection have been modelled extensively. Most models address either of the two hypotheses, and are so-called “top-down” models. Assuming that heterozygous individuals have a higher fitness than homozygous individuals (see for example [212]), or assuming that individuals carrying rare alleles have a higher fitness than individuals carrying common alleles (see for example [212, 230, 231]), it has been shown that an existing MHC polymorphism can be maintained.

Here we take a more mechanistic approach by making no assumptions about selective advantages or disadvantages. We develop a computer simulation to study the coevolution of diploid hosts with haploid pathogens. Our model allows us to study the effect of selection for heterozygosity and frequency-dependent selection on the polymorphism of MHC molecules, by comparing simulations in which pathogens do coevolve with simulations in which they do not. Starting from a population diversity of only one MHC molecule, we show that a diverse set of functionally different MHC molecules is obtained. Our analysis demonstrates that selection involving rapid evolution of pathogens can account for a much larger MHC diversity than selection for heterozygosity alone can.

## Simulating the coevolution of hosts and pathogens

We have developed a genetic algorithm [96] to investigate the coevolution of pathogens and MHC molecules. Genetic algorithms are frequently applied as problem-solving tools, using the principles of evolution to find solutions in for example optimization problems. Instead, we use them here as a simulation of evolution (see also [79, 161]), and thereby take them “right back to where they started from” [103].

In our simulations, we consider a population of  $N_{\text{host}}$  diploid hosts, each represented by a series of bit strings coding for two alleles at  $N_L$  MHC loci. Pathogens are haploid and occur in  $N_S$  independent species of maximally  $N_G$  different genotypes. For simplicity, we omit the complex process of protein degradation into peptides, and model each pathogen by  $N_P$  bit strings representing the set of peptides that can possibly be recognized by a host. Peptide presentation by an MHC molecule can occur at different positions on the MHC molecule, and is modelled by complementary matching. Peptides are  $L_P$  bits long, and MHC molecules are  $L_M$  bits long. For each peptide of a pathogen we seek for each MHC molecule of a host the position at which the peptide finds the maximal complementary match. If the number of complementary bits at this position is at least a predefined threshold  $L_T$ , the peptide is considered to be presented by that particular MHC molecule. In the simulations presented here, pathogens consist of  $N_P = 20$  different peptides, which are  $L_P = 12$  bits long. MHC molecules are  $L_M = 35$  bits long, and present a peptide if at least  $L_T = 11$  out of 12 peptide bits match with the MHC. Thus, the chance that a random MHC molecule presents a randomly chosen peptide is  $7.3\%$ <sup>1</sup>, and the chance that a pathogen of  $N_P = 20$  peptides escapes presentation by a randomly chosen MHC molecule is  $P_e = 22\%$ . Hosts carrying different MHC molecules will hence typically present different peptides of the pathogens.

The quality of different MHC molecules varies. Some MHC molecules may be more stably expressed on the surfaces of host cells than others, or fold into a better peptide-binding groove. To model such MHC differences, a random quality parameter  $0 < Q < 1$  (drawn from a uniform distribution) is attributed to every MHC molecule in the population. These quality differences between MHC molecules prevent extensive drift in simulations with random pathogens. The fitness contribution of a host–pathogen interaction is determined by the quality of the best MHC molecule that is able to present a peptide of the pathogen. We omit the role of lymphocytes by assuming that every peptide that gets presented is recognized by at least one functional clonotype. The role of lymphocytes, and in particular the (functional) deletion of lymphocytes during self tolerance induction, is studied in a follow-up paper (work in progress, see also [34]).

At each generation, every host interacts with every genotypically different pathogen. To account for the shorter generation time of pathogens, we can allow for several pathogen

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<sup>1</sup>The chance that a random peptide binds at a random, predefined position of an MHC molecule is  $P_b = \sum_{i=L_T}^{L_P} \binom{L_P}{i} (0.5)^{L_P}$ . Thus, the chance that a random MHC molecule presents a randomly chosen peptide is  $1 - (1 - P_b)^{L_M - L_P + 1} = 7.3\%$ .

generations per host generation. The fitness  $f_h$  of a host is proportional to the fraction of pathogens it is able to present:

$$f_h = \sum_{i=1}^{N_{\text{path}}} Q_i / N_{\text{path}} \quad (1)$$

where  $N_{\text{path}}$  denotes the total number of different genotypes in the pathogen populations.  $Q_i$  denotes the quality of the best MHC molecule presenting at least one peptide of pathogen  $i$ ; we set  $Q_i$  to zero if none of the MHC molecules of a host present pathogen  $i$ . Similarly, the fitness  $f_p$  of a pathogen is proportional to the fraction of hosts that the pathogen can infect without being presented on the host's MHC molecules:

$$f_p = 1 - \sum_{j=1}^{N_{\text{host}}} Q_j / N_{\text{host}} \quad (2)$$

where  $Q_j$  is the quality of the best MHC molecule of host  $j$  that presents at least one peptide of the pathogen. Again,  $Q_j$  is set to zero if none of the MHC molecules of host  $j$  present the pathogen.

At the end of each generation all individuals are replaced by fitness-proportional reproduction. The sizes of the host population and all pathogen species remain constant. All fitnesses are rescaled such that the highest fitness in each population/species becomes one and the lowest becomes zero. The different individuals in the host population, and the different genotypes in each pathogen species reproduce according to a fitness-dependent reproduction function:

$$P_r(i) = \frac{e^{s\bar{f}_i}}{\sum_{j=1}^n e^{s\bar{f}_j}}, \quad (3)$$

where  $P_r(i)$  is the reproduction probability of host  $i$  or pathogen genotype  $i$ ,  $\bar{f}_i$  denotes its rescaled fitness, and  $n$  is the total number of different individuals in the host population or genotypes in the particular pathogen species. In our simulations, the selection coefficient  $s$  is set to one so that the reproduction chance of the fittest individual is 2.73 times higher than that of the individual with the lowest fitness. Pathogen genotypes reproduce asexually; new-born pathogens come from parents of the same pathogen species. New-born hosts have two parents, each of which donates a randomly selected MHC allele. During reproduction point mutations can occur. Both peptides and MHC molecules have a mutation chance of  $\mu = 0.1\%$  per bit per generation. The chance for a new-born host to receive a nonmutated MHC molecule is thus  $(1 - \mu)^{L_M} = 96.6\%$ , and the chance for a new-born pathogen to receive a nonmutated peptide is  $(1 - \mu)^{L_P} = 98.8\%$ . One cycle of fitness determination, reproduction, and mutation defines a generation. We study evolution over many generations.

## A dynamically maintained polymorphism

The simulation model allows us to study the mutual influence of host and pathogen co-evolution on the composition of MHC molecules in the host population, and peptides in the pathogen species. In particular, we will (i) study whether a polymorphic set of MHC molecules can develop from an initially nondiverse host population, and (ii) investigate the relative roles of frequency-dependent selection and selection for heterozygosity, in maintaining the polymorphism of MHC molecules.

All simulations are initialized with random pathogen genotypes, and all hosts initially carry identical MHC molecules, *i.e.* there is neither variation between MHC molecules within the hosts, nor between the hosts. Two examples of such simulations are shown in Figure 1, in which the average fitnesses of the pathogens and the hosts are plotted as a function of the host generation  $t$ . To study the effect of the typically short generation time of pathogens, we consider two different cases. In one of them (Figures 1*a* and *b*) the pathogens evolve just as fast as the hosts, while in the other case (Figures 1*c* and *d*) the pathogens evolve one hundred times faster than the hosts. Since there is no initial MHC diversity, the pathogens immediately attain a relatively high fitness, and the hosts a correspondingly low fitness, in both simulations. Any pathogen that is able to infect one host is able to infect all hosts, and hence rapidly takes over the pathogen population. Under this selective pressure caused by the pathogens, the hosts develop an MHC polymorphism (as will be shown in the next section), and in so doing regain a high fitness. After about 300 host generations a quasi-equilibrium is approached which is followed until generation  $t = 1000$ . A similar equilibrium is attained if the host population is initialized with random MHC molecules (not shown). The average fitnesses during the quasi-equilibrium depend on the relative generation time of the pathogens. The faster the pathogens evolve, the higher their average fitness, and the lower the average fitness of the hosts (Figure 2). Once the pathogens evolve one hundred times faster than the hosts, the average pathogen fitness saturates.

The quasi-equilibrium that is approached is a dynamic one. As in a *Red Queen* situation, hosts and pathogens are continually counteracting each other by adaptation. This follows from additional simulations in which from  $t = 1000$  onwards, further evolution of either the hosts or the pathogens is prevented. If the pathogens and the hosts evolve equally fast, and the evolution of the hosts is subsequently halted, the pathogens markedly increase their fitness (Figure 1*a*). Such an increase of the average pathogen fitness is not observed, however, if the pathogens were evolving one hundred times faster than the hosts before the evolution of the hosts was stopped (Figure 1*c*). Their short generation time apparently enabled the pathogens to “completely” adapt during each host generation even before the host population was frozen. Stopping the evolution of the hosts then hardly makes a difference. Remarkably, once the evolution of the hosts is stopped, the pathogens that used to evolve as fast as the hosts attain a significantly higher average fitness (Figure 1*a*) than the pathogens that used to evolve faster than the hosts (Figure 1*c*). The reason for this difference will be addressed in the next section.

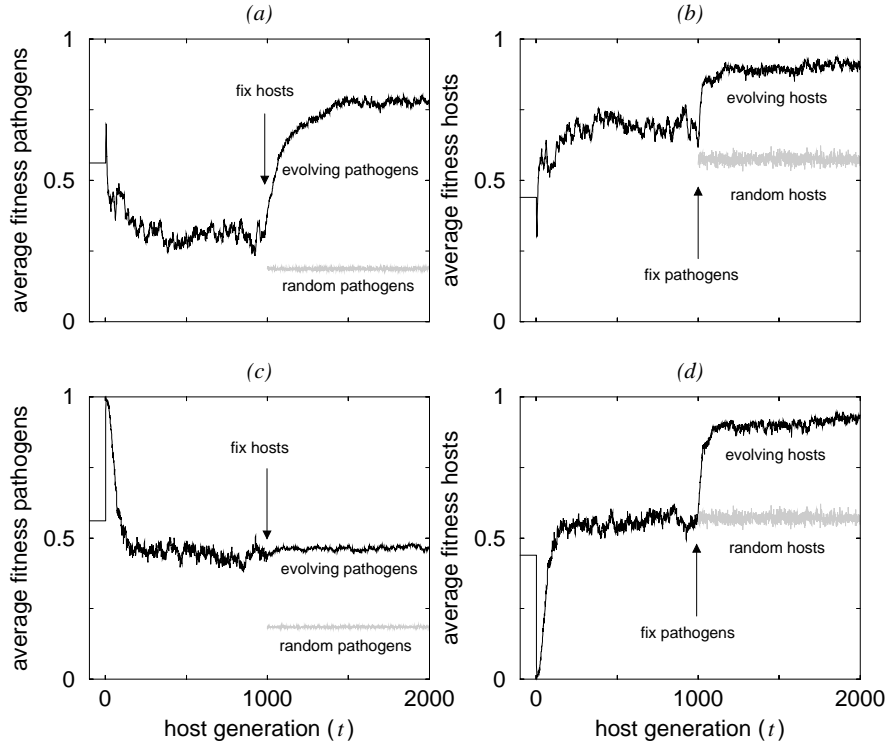


Figure 1. The average fitnesses of pathogens (*a, c*) and hosts (*b, d*) in a simulation in which the pathogens evolve as fast as the hosts (*a, b*), and a simulation in which the pathogens evolve one hundred times faster than the hosts (*c, d*), plotted against the host generation  $t$ . Note that by equations (1) and (2), the average host and pathogen fitnesses in a single simulation always sum up to one. The simulations are initialized with MHC-identical hosts and random pathogens. Coevolution is stopped at host generation  $t = 1000$ . We either stop the evolution of the hosts, and let only the pathogens go on evolving (*a, c*), or we stop the evolution of the pathogens, and let only the hosts go on evolving (*b, d*). The grey lines denote the average fitness of randomly created pathogens evaluated on the fixed host populations of generation  $t = 1000$  (*a, c*), and the average fitness of random, heterozygous hosts evaluated on the fixed pathogen populations of generation  $t = 1000$  (*b, d*). Parameters are:  $N_{\text{host}} = 200$ ,  $N_L = 1$ ,  $N_S = 50$ ,  $N_G = 10$ ,  $N_P = 20$ ,  $L_P = 12$ ,  $L_M = 35$ ,  $L_T = 11$ .

Likewise, if the evolution of the pathogens is stopped and only the hosts go on evolving, the hosts evolve such that they can resist almost all pathogens, *i.e.* they approach fitness one (Figures 1*b* and *d*). Pathogens that evolve in a non-evolving host population attain a larger average fitness than random pathogens (see the grey lines in Figures 1*a* and *c*). Similarly, evolving hosts in the presence of a non-evolving pathogen population attain a higher fitness than random, heterozygous hosts (see the grey lines in Figures 1*b* and *d*). Thus, evolving hosts and pathogens have the capacity to adapt to non-evolving populations of pathogens or hosts, respectively.

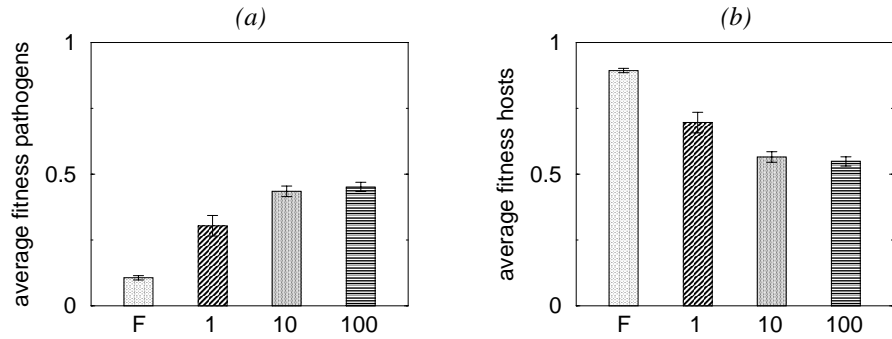


Figure 2. The average fitnesses of pathogens (a) and hosts (b) over the last one hundred generations of the coevolution (*i.e.* between  $t = 900$  and  $t = 1000$ ). Results are shown for four different simulation types: F = fixed (non-evolving) pathogens, 1 = pathogens evolving as fast as the hosts, 10 = pathogens evolving ten times faster than the hosts, 100 = pathogens evolving one hundred times faster than the hosts. In the coevolutionary simulations there are typically two different genotypes per pathogen species (not shown). We therefore initialized the F simulation with two randomly chosen pathogen genotypes per species. The error bars denote the standard deviations of the average host and pathogen fitnesses in time. For parameters see the legend of Figure 1.

## Host and pathogen evolution

As soon as a coevolutionary simulation is started, the number of different MHC molecules in the host population rapidly increases to reach a high quasi-equilibrium diversity (see Figure 3). This diversification also occurs if the pathogens do not evolve at all. In that case, the high population diversity of MHC molecules is due to selection favouring heterozygous hosts. The faster the pathogens evolve, however, the larger the MHC population diversity becomes (see Figure 4a).

To check if the MHC molecules arising in a host population are really different from each other, and do not differ at a few mutations only, we have calculated the average genetic distance (Hamming distance) between all different MHC molecules in the host population (Figure 4b). Evolution of the pathogens appears to increase MHC diversity; the shorter the generation time of the pathogens, the larger the genetic distance between the MHC molecules of the hosts. Thus, rapidly coevolving pathogens trigger selection for a functionally diverse set of MHC molecules.

In order to measure to what extent the pathogens evade presentation on the MHC molecules of the hosts, we have calculated the average fraction of peptides from the pathogen genotypes that is presented by the MHC molecules in the host population. The faster the pathogens evolve, the better their evasion of presentation by the hosts' MHC molecules (see the patterned bars in Figure 5). If the pathogens evolve, the average fraction of peptides that is presented by the MHC molecules of the hosts is smaller than the



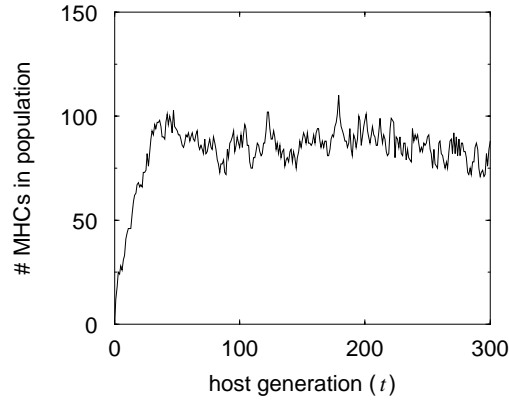


Figure 3. Evolution of MHC polymorphism. The number of different MHC molecules in the host population plotted from the start of the coevolution ( $t = 0$ ) until host generation  $t = 300$ . The generation time of the pathogens is one hundred times shorter than that of the hosts. For parameters see the legend of Figure 1.

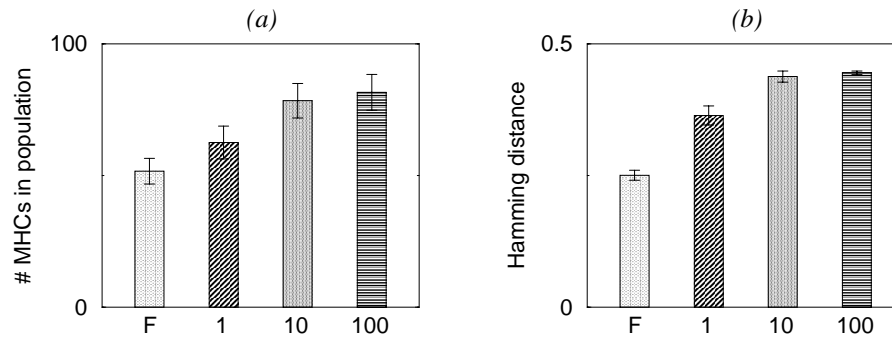


Figure 4. MHC molecules become functionally polymorphic. (a) The average number of different MHC molecules in the host population. (b) The average of the Hamming distances between all possible pairs of different MHC molecules in the host population. For parameters and axis labels see the legends of Figures 1 and 2.

expected 7.3% that we calculated above for MHC molecules binding random peptides. Thus, the pathogens in our simulations indeed evolve towards evasion of presentation by the particular MHC molecules that are present in the host population.

We have applied a similar analysis to the simulations in which either the hosts or the pathogens are prevented from evolving. This analysis partially explains our earlier observation that pathogens evolving in a frozen host population that has been stringently selected by rapidly coevolving pathogens (Figure 1c) attain a lower fitness than pathogens evolving in a host population that has been selected only moderately (Figure 1a). If the pathogens do not evolve faster than the hosts, the fraction of pathogen peptides recognized by the hosts' MHC molecules decreases dramatically when the evo-

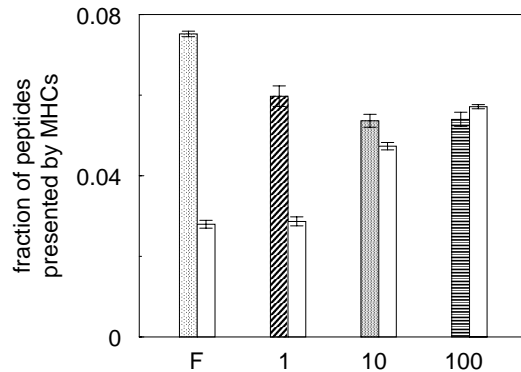


Figure 5. Pathogens evolve towards evasion of presentation by the particular MHC molecules that are present in the host population. The average presentation efficiency of the MHC molecules, *i.e.* the average fraction of peptides from the pathogen genotypes that is presented by the MHC molecules, is plotted for different pathogen generation times. The patterned bars denote the average presentation efficiency of the MHC molecules of coevolving hosts, *i.e.* between host generation  $t = 900$  and  $t = 1000$  in Figure 1. The white bars denote the average presentation efficiency of the MHC molecules that have been frozen at host generation  $t = 1000$  in Figure 1, after the pathogens have been allowed to evolve for one thousand generations, *i.e.* between host generation  $t = 1900$  and  $t = 2000$  in Figure 1. For parameters and axis labels see the legends of Figures 1 and 2.

lution of the hosts is stopped (see the white bars denoted by F and 1 in Figure 5). Apparently, during the coevolution the hosts specialized on the particular pathogens that were present in the population. This specialization enables the pathogens to escape immune recognition once the evolution of the hosts is stopped. In contrast, if the pathogens evolve faster than the hosts during the coevolution, the hosts cannot specialize on the particular pathogens that are present in the population. As a consequence, the pathogens fail to escape immune recognition once the evolution of the hosts is stopped (see the white bars denoted by 10 and 100 in Figure 5). Another reason why the evolutionary history of a frozen host population influences the escape possibilities of a pathogen lies in the polymorphism of the hosts' MHC molecules. As we have seen above, the faster the evolution of the pathogens is, the more polymorphic the MHC molecules of the hosts become. Thus, pathogens evolving in a frozen host population that used to be stringently selected by rapidly coevolving pathogens, have more difficulty in escaping presentation by the highly polymorphic MHC molecules of the hosts.

## Heterozygosity versus frequency-dependent selection

Since the evolution of pathogens can be switched off in our model, we can separately study the effect of selection for heterozygosity. In coevolutionary simulations there will

be selection for heterozygosity, as well as frequency-dependent selection. One possibility to exclude evolution of the pathogens is to let the hosts evolve in response to a fixed pathogen population. As we have seen, in that case hosts adapt to the specific pathogens that are present (Figure 5). To exclude this specialization we have also performed simulations in which at every host generation all pathogens are replaced by random ones (denoted by R in the figures).

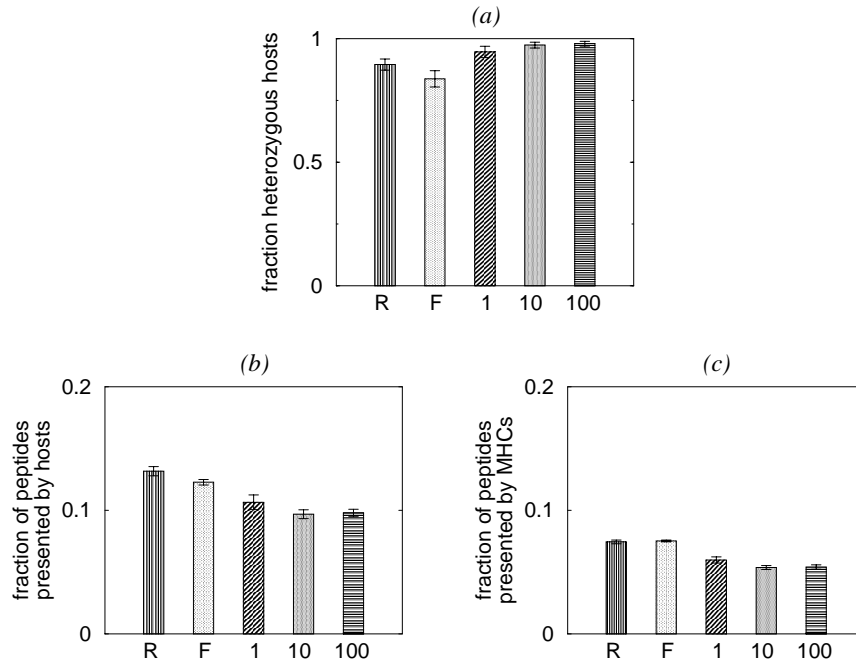


Figure 6. Hosts become functionally heterozygous. (a) The average fraction of heterozygous hosts. (b) The average fraction of peptides from the pathogens presented by the hosts. (c) The average fraction of peptides from the pathogens presented by the individual MHC molecules of the hosts. R denotes the simulation in which pathogens are introduced randomly at every host generation. Like the fixed pathogen population denoted by F, randomly introduced pathogen species consist of two randomly created pathogen genotypes per species. For parameters and axis labels see the legends of Figures 1 and 2.

The role of selection for heterozygosity appears to be strong under all conditions. During the quasi-equilibrium, the fraction of heterozygous hosts is always close to one (Figure 6a). To check if this heterozygosity is also functional, *i.e.* if the two MHC molecules of a host are generally presenting different peptides, we compare the average fraction of peptides from the pathogens that are presented by the hosts (Figure 6b), with the average fraction of peptides from the pathogens presented by their individual MHC molecules (Figure 6c). It appears that in all simulations, the hosts (with their two MHC molecules) present nearly twice as many peptides as their individual MHC molecules. Thus, the hosts in our simulations indeed typically carry functionally different MHC molecules.

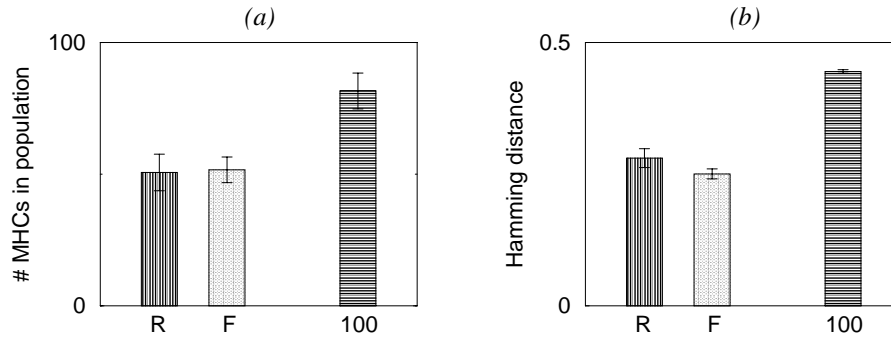


Figure 7. Selection for heterozygosity versus frequency-dependent selection. (a) The average number of different MHC molecules in the host population, and (b) the average Hamming distance between the different MHC molecules. We have plotted a coevolutionary simulation in which the pathogens evolve one hundred times faster than the hosts (100), and two simulations in which the pathogens do not evolve (R and F). The coevolutionary simulation represents the MHC diversity that evolves in the presence of both frequency-dependent selection and selection for heterozygosity, while the two latter simulations (R and F) represent the MHC diversity that evolves under selection for heterozygosity only.

In order to study the relative roles of selection for heterozygosity and frequency-dependent selection, we compare the MHC polymorphism arising in the absence and presence of frequency-dependent selection. Figure 7a shows that heterozygosity plus frequency-dependent selection (*i.e.* a simulation with evolving pathogens, denoted by 100) results in a much higher degree of polymorphism than selection for heterozygosity alone (*i.e.* simulations with non-evolving pathogens, denoted by R and F). The average genetic differences between the MHC molecules that arise support this notion (see Figure 7b). Summarizing, our simulations show (i) that a polymorphic set of MHC molecules rapidly develops in an initially nondiverse host population, and (ii) that selection by coevolving pathogens can account for a much larger population diversity of MHC molecules than mere selection for heterozygosity can.

## Discussion

We have shown that both the origin and the maintenance of MHC polymorphism can be understood in a model that does not assume any *a priori* selective advantage of heterozygous hosts or hosts with rare MHC molecules. By starting our simulations with MHC-identical hosts, we have in fact studied a “worst case” scenario. Polymorphisms of MHC-like molecules seem to have been present since colonial or multicellular life [43]. Thus, the *origin* of MHC polymorphism may not lie in immune function. De Boer [61] for example showed that in primitive colonial organisms the preservation of “genetic identity” is sufficient to account for highly polymorphic histocompatibility molecules.

Our simulation model demonstrates that coevolution of hosts and pathogens yields a larger MHC polymorphism than merely selection for heterozygosity. Our analysis thus supports the view that additional selection pressures on top of overdominant selection do play a role in the evolution of the MHC polymorphism [165, 230]. It has been shown experimentally that many MHC alleles have persisted for significant evolutionary periods of time [112, 113, 118, 136]. This has been used as an argument against frequency-dependent selection [99], but was later demonstrated to be compatible with selection for rare MHC molecules [212]. Analysis of the persistence of particular MHC alleles in our simulations would allow to study this in more detail.

In order to increase the speed of our simulations, we have used a rather high mutation frequency of the hosts' MHC molecules, *i.e.*  $\mu = 0.001$  per bit per generation. Decreasing this mutation frequency indeed resulted in a lower MHC population diversity. Increasing the host population size in our simulations, on the other hand, increased the MHC polymorphism. Using a mutation frequency of MHC molecules of  $\mu = 10^{-6}$  and a host population size of  $N_{\text{host}} = 1000$  hosts, we still found a population diversity of approximately 30 different MHC molecules. Independently of the choices of  $\mu$  and  $N_{\text{host}}$ , the MHC polymorphism that was attained in coevolutionary simulations was always considerably (*e.g.* 5-fold) higher than the polymorphism arising under overdominant selection only (results not shown).

Regarding the enormous population diversity of MHC molecules observed in nature [166, 223], it is surprising that the number of different MHC molecules expressed per individual is quite limited [167]. In our simulations hosts carry only one MHC gene. What would change if this number of MHC genes per individual would be increased? Individuals expressing more MHC genes would be expected to have a selective advantage, in that more pathogens would be presented. This selective advantage would vanish, however, once the chance to present (at least one peptide from) any pathogen approaches 100%. For the parameter setting used here, the chance that a random pathogen consisting of twenty peptides evades presentation by a single MHC molecule is  $P_e = 22\%$ . In the absence of pathogen evolution, expression of about ten different MHC molecules would thus be sufficient to ensure the presentation of virtually any pathogen. In coevolutionary situations, however, the selection for expression of MHC molecules that are different from the other MHC molecules in the population would remain. This selection will only disappear when the number of different MHC molecules per individual becomes so large that every host is expected to present all pathogen peptides. If individuals no longer draw different "samples" from the pool of peptides from each pathogen, pathogens may be expected to exploit this "predictability" of the hosts' immune responses [231]. In Chapter 4, for instance, we have demonstrated that increasing the number of MHC loci increases the likelihood of autoimmunity [35]. Extension of the current model with host self molecules and a variable number of MHC genes may shed light on the role of such mechanisms in the maintenance of the MHC polymorphism (work in progress).



# 6

## **Extending the quasi-steady state approximation by changing variables**

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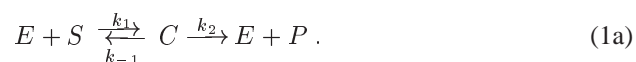
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## Abstract

The parameter domain for which the quasi-steady state assumption is valid can be considerably extended merely by a simple change of variable. This is demonstrated for a variety of biologically significant examples taken from enzyme kinetics, immunology, and ecology.

## Introduction

Prototypical in biochemistry is the reversible reaction between enzyme  $E$  and substrate  $S$  to give complex  $C$ , which irreversibly yields product  $P$ :



The classical Michaelis–Menten approximation for scheme (1a) is the archetypal example for the use of the quasi-steady state (QSS) approximation. This approximation is a major simplifying step throughout biology, with its enormous range of time scales, and indeed in many other branches of science. The quasi-steady state assumption (QSSA) often yields revealing analytic formulas and it frequently circumvents problems of stiffness in the numerical integration of systems of differential equations. It is thus of considerable utility to be able to characterize parameter domains wherein a QSSA provides a valid approximation.

Virtually all biochemistry texts discuss the application of the QSSA to scheme (1a) and its consequences, such as the use of the Lineweaver–Burk plot to obtain the maximum velocity of the reaction  $V_{\max}$  and the Michaelis constant  $K_m$ . As far as we know, none of these texts indicates conditions under which the QSSA should be valid. Such conditions do appear in the literature, but only rather recently has it been shown [163, 189, 192] that the usually cited requirement  $E_0 \ll S_0$  is too strong; the classical QSSA is in fact valid providing that

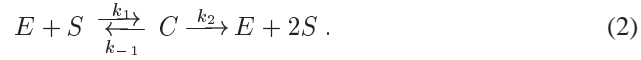
$$E_0 \ll S_0 + K_m , \quad (1b)$$

where  $K_m$  is the Michaelis constant. See the derivation of condition (11) below.

This chapter examines scheme (1a) when there is an excess of enzyme, so that condition (1b) does not hold. The classical QSSA breaks down in these situations, which can be encountered *in vivo* [203, 206] or in biotechnological applications. Remarkably, a simple change of variable permits the validity of the classical QSSA to be considerably extended so that the new situations are covered in many instances.



We show that “enzyme excess” arises naturally in schemes of the form



One example of scheme (2) is the proliferation of T cells in response to antigen. Then  $S$  denotes a replicating T cell,  $E$  a site on an antigen-presenting cell (APC), and  $C$  a complex of a T cell bound to an APC. This scheme has been analysed by De Boer & Perelson [63] employing the classical Michaelis–Menten approach. We here improve upon this earlier analysis. Another instance of scheme (2) is one wherein  $E$  is a catalytic RNA molecule while  $S$  is another RNA molecule whose replication is catalysed by  $E$  [72].

A major point of this chapter is to show that, paradoxically, the standard QSSA for the complex  $C$  in fact remains valid in a parameter domain that overlaps condition (1b), but also considerably extends condition (1b), provided merely that the free substrate concentration  $S$  is replaced by the total substrate concentration  $\bar{S} \equiv S + C$ . In pursuing this goal, we employ and extend earlier considerations concerning the validity of the QSSA. Thus this chapter also serves as a brief and up to date primer and case study on how to estimate when the QSSA is applicable.

After discussing the “total QSSA” for the prototype example of scheme (1a), we present several models where the same basic idea leads to useful new approximate solutions: models for replication schemes such as scheme (2) and for certain predator–prey interactions.

## The standard QSSA and its limits of validity

Differential equations corresponding to scheme (1a) can be written as

$$\frac{dS}{dt} = -k_1(E_0 - C)S + k_{-1}C, \quad (3a)$$

$$\frac{dC}{dt} = k_1[(E_0 - C)S - K_m C], \quad (3b)$$

where

$$K_m = \frac{k_{-1} + k_2}{k_1}. \quad (4)$$

The conservation law

$$E + C = E_0 \quad (5a)$$

yields  $E$ . Initial conditions are

$$S(0) = S_0, \quad (5b)$$

$$C(0) = 0, \quad (5c)$$

$$E(0) = E_0, \quad (5d)$$

$$P(0) = 0. \quad (5e)$$

(Note that the choice  $P(0) = 0$  does not influence  $S$ ,  $E$  or  $C$ .)

In the standard approach, one assumes that after a fast transient,  $C$  can be regarded as in equilibrium. From  $dC/dt = 0$  it follows that

$$C = \frac{E_0 S}{K_m + S}. \quad (6)$$

A differential equation for  $S$ , valid after the transient, can be most easily derived by realizing that if  $dC/dt$  is effectively zero, then equation (3b) can be added to equation (3a), yielding

$$\frac{dS}{dt} = -k_2 C, \quad (7a)$$

i.e.

$$\frac{dS}{dt} = -\frac{k_2 E_0 S}{K_m + S}. \quad (7b)$$

It is conventional to assume that the substrate level changes negligibly during the fast transient so that equation (5b) can also serve as an “initial condition” for equation (7b). Differential equation (7b) and initial condition (5b) thus constitute the QSSA. One hopes that this QSSA will provide a good approximation for calculating the post-transient development of the system under consideration.

According to the procedure described by Segel [189], the first step in attempting to determine the parameter ranges for which the QSSA is valid is to estimate two time scales. These are  $t_C$ , the time that characterizes the duration of the fast transient, and  $t_S$ , the magnitude of time required for a significant change in  $S$  during the post-transient period. To estimate the fast time scale  $t_C$  we model the initial rapid accumulation of  $C$  by substituting  $S = S_0$  in equation (3b). This transforms equation (3b) into a linear equation of which

$$t_C = \frac{1}{k_1(S_0 + K_m)} \quad (8)$$

is the time scale. Another way to obtain the same estimate of  $t_C$  is to realize (from equation (6) with  $S = S_0$ ) that during the fast transient the total change of the complex is approximately  $\Delta C = E_0 S_0 / (K_m + S_0)$ . At the maximum rate at which the complex increases (see equation (3b)) this indeed takes  $t_C = \Delta C / (k_1 E_0 S_0)$  time units.

To estimate the slow (substrate) time scale  $t_S$  we consider  $dS/dt$  after the fast transient, *i.e.* equation (7b). We employ the characterization of  $t_S$  that was suggested by Segel [188], *i.e.* we take the maximum post-transient change in substrate,  $S_0$ , divided by the post-transient maximum of  $|dS/dt|$ , obtained by substituting  $S = S_0$  in equation (7b). Thus we write

$$t_S = \frac{S_0}{|dS/dt|_{\max}}, \quad (9a)$$

*i.e.*

$$t_S = \frac{K_m + S_0}{k_2 E_0}. \quad (9b)$$

The first condition necessary for the QSSA is that the fast time scale  $t_C$  is indeed much smaller than the slow time scale  $t_S$ . This yields

$$\frac{k_2 E_0}{k_1 (S_0 + K_m)^2} \ll 1. \quad (10)$$

Secondly, to insure that equation (5b) can be taken as an “initial condition,” we require that there be a small fractional depletion of substrate during the initial transient. This is ensured by demanding that the fractional change  $|\Delta S/S_0|$  is small during the fast transient. We overestimate  $\Delta S$  by the product of the maximum rate of depletion of  $S$ , *i.e.* (from equation (3a))  $k_1 E_0 S_0$ , with the duration of the fast transient  $t_C$ . From this we find that the condition  $|\Delta S/S_0| \ll 1$  requires

$$\frac{E_0}{S_0 + K_m} \ll 1. \quad (11)$$

Since condition (10) can be written

$$\frac{E_0}{S_0 + K_m} \frac{1}{1 + (k_{-1}/k_2) + (S_0 k_1/k_2)} \ll 1$$

we see that condition (11) is stronger than condition (10). Thus, condition (11) guarantees the accuracy of the classical QSSA.

## The effects of replacing free by total substrate concentration

In conditions of enzyme excess, when condition (11) and hence the standard QSSA are not expected to be valid we introduce the *total substrate concentration*  $\bar{S}$ , where

$$\bar{S} \equiv S + C. \quad (12)$$

$\bar{S}$  rather than  $S$  will now be our substrate variable. The total substrate  $\bar{S}$  cannot be depleted by the formation of complex. Because the validity of the classical QSSA depends strongly on negligible initial depletion of substrate (see the derivation of equation (11)),

this simple variable change is expected to have an important effect. We derive the conditions for the QSSA of this redefined “total substrate” model, which we term the tQSSA.

If we substitute  $\bar{S}$  for  $S$  the governing equations become

$$\frac{d\bar{S}}{dt} = -k_2 C, \quad (13a)$$

$$\frac{dC}{dt} = k_1 [(E_0 - C)(\bar{S} - C) - K_m C], \quad (13b)$$

with initial conditions

$$\bar{S}(0) = S_0, \quad (14a)$$

$$C(0) = 0. \quad (14b)$$

Assuming  $dC/dt = 0$  as before, but now using equation (13b), we find that  $C$  should be replaced by a solution of the quadratic

$$C^2 - (E_0 + K_m + \bar{S})C + E_0\bar{S} = 0. \quad (15)$$

The constraint  $C \leq E_0$ , which follows from the conservation law (5a) and the non-negativity of  $E$ , implies that equation (15) has a unique solution. (One takes the negative square root in the quadratic formula.)

Quadratics such as equation (15) are common in kinetic calculations. For use in further kinetic manipulations, it is helpful to replace the somewhat complicated analytic formula for the solution of the quadratic by a simpler and more transparent expression. (This step is not essential to our main line of argument.) Such an expression is provided by a two-point Padé approximant [15]. The lowest order Padé approximant consists of the quotient of two linear functions of  $\bar{S}$ . Around  $\bar{S} = 0$  and for  $\bar{S} \rightarrow \infty$  we approximate equation (15) by

$$C = \frac{E_0\bar{S}}{E_0 + K_m} \quad (16a)$$

and

$$C = E_0, \quad (16b)$$

respectively. Thus the Padé approximant is

$$C = \frac{E_0\bar{S}}{E_0 + K_m + \bar{S}}. \quad (17)$$

Formula (17) can also be obtained by neglecting the  $C^2$  term in equation (15). This is consistent if for any value of  $\bar{S}$ ,

$$C = \frac{E_0\bar{S}}{E_0 + K_m + \bar{S}} \ll E_0 + K_m + \bar{S}, \quad (18)$$

i.e. if for any value of  $\bar{S}$ ,

$$1 \ll \left(1 + \frac{\bar{S}}{E_0} + \frac{K_m}{E_0}\right) \left(1 + \frac{E_0}{\bar{S}} + \frac{K_m}{\bar{S}}\right). \quad (19)$$

This certainly holds if  $\bar{S}$  is either large or small compared to  $E_0$ . Even if  $\bar{S} = E_0$ , the right side of condition (19) is not less than  $(2 + K_m/E_0)^2$ . Thus we conclude that neglecting the  $C^2$  term in equation (15) to obtain equation (17) is indeed justified. Cha & Cha [48] and Cha [47] developed another method for deriving equation (17) as an approximation of equation (15). Cha [47] numerically shows that the approximation is generally good.

To obtain an estimate for the range of validity of the new tQSSA, let us first estimate the time scale for the fast transient. During this period the complex concentration  $C$  begins from an initial value of zero and remains relatively small. Thus, again neglecting the terms quadratic in  $C$  when adapting equation (13b) for our present purposes, we obtain

$$\frac{dC}{dt} = k_1 [E_0 S_0 - (E_0 + S_0 + K_m)C]. \quad (20)$$

In equation (20) we have made the simplifying approximation, which should be well warranted during the brief transient, that  $\bar{S}(t) \approx S_0$ . From the solution of equation (20) it is clear that

$$t_C = \frac{1}{k_1(E_0 + S_0 + K_m)}. \quad (21)$$

Note that  $t_C$  can again be derived by calculating  $\Delta C/|dC/dt|_{\max}$  as outlined just below equation (8).

It is at first surprising that the time scale  $t_C$  in equation (21) is not the same as its counterpart in equation (8). The “ $\Delta C$ ” approach to calculating  $t_C$  leads to the realization that the different values of  $t_C$  can be traced to the different expressions of equations (6) and (17) for  $C$ . However, for equation (6) to be valid then it is necessary that  $E_0 \ll S_0 + K_m$  (condition (11)). If this is the case then the  $E_0$  term is negligible in equation (17), at least until the considerable time elapses for  $\bar{S}$  to drop below  $S_0$ . Additionally, if the  $E_0$  term is negligible, the alternative formulas for  $C$  and for  $t_C$  are in fact identical. Thus whenever the alternatives of the classical QSSA given by equations (8) and (6) are valid they give answers that are indistinguishable from the tQSSA counterparts given by equations (21) and (17); only when equations (8) and (6) are invalid are they truly different from equations (21) and (17).

In order to estimate the slow time scale  $t_{\bar{S}}$  we again consider the maximum change of  $\bar{S}$  divided by the maximum rate of change of total substrate after the fast transient. From equation (13a) with  $C$  given by the Padé approximant of equation (17), and with  $\bar{S} = S_0$ , calculation of  $S_0/|d\bar{S}/dt|_{\max}$  yields

$$t_{\bar{S}} = \frac{E_0 + S_0 + K_m}{k_2 E_0}. \quad (22)$$

The necessary condition for the validity of the tQSSA,  $t_C \ll t_{\bar{S}}$ , thus takes the following form:

$$\frac{k_2 E_0}{k_1 (E_0 + S_0 + K_m)^2} \ll 1. \quad (23)$$

To check that initial condition (14a) is appropriate for our tQSSA, we require that the change of  $\bar{S}$  is small during the fast transient. Paralleling the derivation of condition (11), we multiply the maximal value of  $|\mathrm{d}\bar{S}/\mathrm{d}t|$  with the duration  $t_C$  of the transient. By equation (13a) this maximum rate of change is  $k_2 C_{\max}$ . An upper limit for  $C_{\max}$  during the fast transient is the QSS value that the complex is approaching. Employing the Padé approximant (equation (17)), and substituting  $\bar{S} = S_0$  (which is also an overestimate), we estimate  $C_{\max}$ . With this we find that condition (23) is also the condition that ensures that  $|\Delta\bar{S}/S_0|$  is small.

Thus condition (23) suffices for the validity of the tQSSA. Below we confirm numerically that if equation (23) is not satisfied we indeed find that (i) after the fast transient, trajectories fail to correspond to the QSSA and also that (ii) during the fast transient, total substrate is depleted by the formation of product.

Finally we observe that we can rewrite equation (23) in the form

$$\left(1 + \frac{E_0 + S_0}{k_2/k_1} + \frac{k_{-1}}{k_2}\right) \left(1 + \frac{S_0 + K_m}{E_0}\right) \gg 1. \quad (24a)$$

The left side of condition (24a) is always greater than unity, so that we expect that the QSSA will always be at least roughly valid. Moreover, there are several different conditions any one of which guarantees that condition (24a) holds. These are

$$E_0 + S_0 \gg k_2/k_1, \quad (24b)$$

$$k_{-1} \gg k_2, \quad (24c)$$

$$S_0 + K_m \gg E_0. \quad (24d)$$

Note from condition (24d) the important finding that when the standard QSSA is valid, then so is the tQSSA. Thus it appears that our new approach considerably extends the parameter range for which a QSSA can be applied.

## Comparing the standard QSSA with the tQSSA

Conditions (11) and (23) can be plotted in parameter space to compare the regions of validity of the QSSA and the tQSSA. Note that this need not be a fair comparison because conditions (11) and (23) need not be equally strong for systems (3) and (13), respectively. Both conditions are of the form  $f(E_0, S_0) \ll 1$ . For definiteness, we draw in

Figure 1 the regions corresponding to  $f(E_0, S_0) \leq 0.1$ . For  $k_1 = 10$ ,  $k_{-1} = 1$ , and  $k_2 = 0.1$  (i.e.  $K_m = 0.11$ ) the standard QSSA is valid in the dotted region in Figure 1a. The tQSSA is valid for any initial condition, by condition (24c). Upon increasing the rate of product formation to  $k_2 = 10$  (i.e.  $K_m = 1.1$ ), a relatively small curved region appears within which the tQSSA is not valid (see Figure 1b; note the change in scale). The (dotted) validity region of the standard QSSA has remained almost the same. Thus Figure 1 illustrates that when the standard QSSA is valid, the tQSSA is valid also. Additionally, the figure suggests that the tQSSA is valid for a much larger domain of initial conditions.

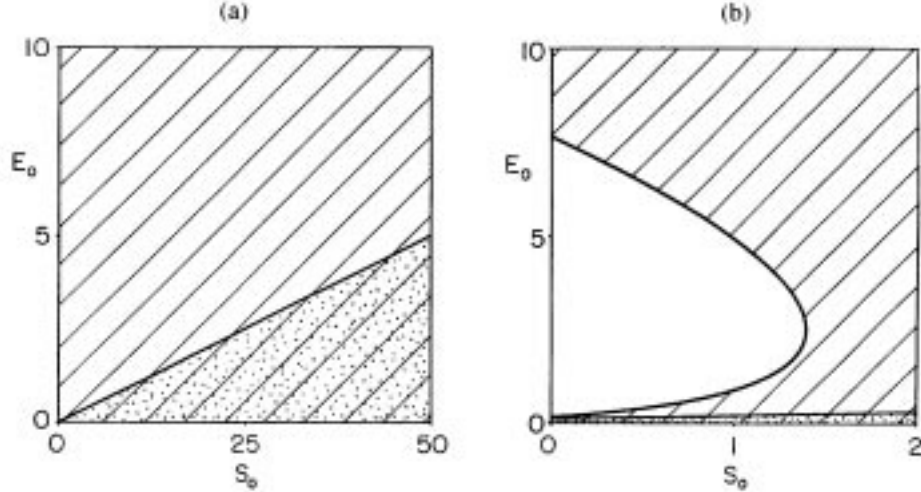


Figure 1. Validity of the QSS and the tQSS assumptions for the standard enzyme–substrate–complex scheme (1a), in the  $E_0$ – $S_0$  plane. Parameters are  $k_1 = 10$  and  $k_{-1} = 1$ . In panel *a*,  $k_2 = 0.1$  ( $K_m = 0.11$ ), while in panel *b*,  $k_2 = 10$  ( $K_m = 1.1$ ). Dotted domain: QSSA valid. Shaded domain: tQSSA valid.

Generally, an analysis like that of Figure 1 can best be performed in terms of dimensionless parameters. We nevertheless show the original parameters because the figure would remain qualitatively the same if we were to employ the following three dimensionless parameters:

$$\eta = \frac{E_0}{K_m}, \quad \sigma = \frac{S_0}{K_m} \quad \text{and} \quad \kappa = \frac{k_2}{k_2 + k_{-1}}. \quad (25a)$$

The dotted and shaded regions in Figure 1 would now, respectively, correspond to

$$\frac{\eta}{1 + \sigma} \leq 0.1 \quad \text{and} \quad \frac{\kappa\eta}{(\eta + \sigma + 1)^2} \leq 0.1, \quad (25b)$$

which have the same form as the curves in Figure 1 when plotted as functions of  $\eta$  and  $\sigma$ . (Increasing  $k_2$  corresponds to increasing  $\kappa$ , which indeed decreases the region in which the tQSSA is valid.) We stress that the results of Figure 1 provide representative examples of the ranges of validity of the QSSA and the tQSSA.

## Numerical confirmation

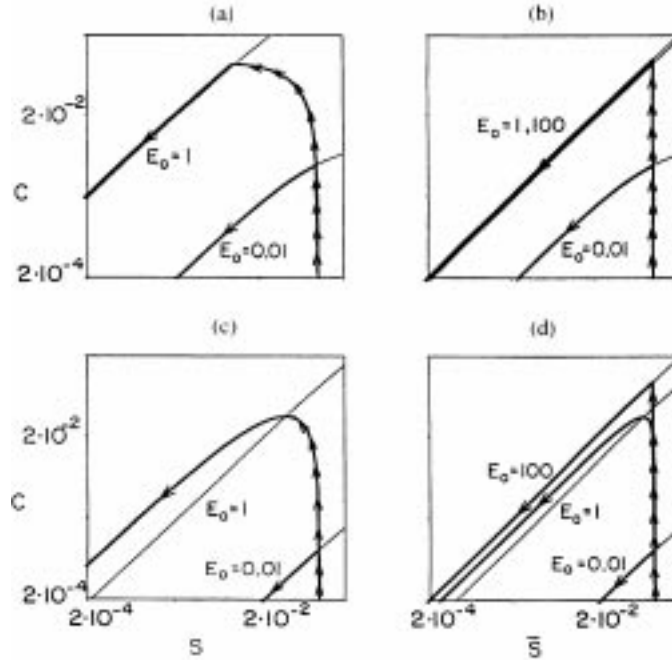


Figure 2. Trajectories approaching the QSS approximation of the product model (equations (3)–(5)) with (panels *b*, *d*) and without (panels *a*, *c*) change of variables from free substrate  $S$  to total substrate  $\bar{S}$ . In the substrate versus complex phase plane the heavy lines depict numerically computed trajectories of the full model for initial conditions  $C(0) = 0$  and  $S(0) = \bar{S}(0) = 0.1$ . The light curves depict the QSS equations for  $C$ , *i.e.* equation (6) or the solution of equation (15). The arrows indicate the direction of the trajectories: a high arrow density implies a relatively rapid traversal. Parameters are  $k_1 = 10$  and  $k_{-1} = 1$ . In panels *a* and *b*,  $k_2 = 0.1$  ( $K_m = 0.11$ ), while in panels *c* and *d*,  $k_2 = 10$  ( $K_m = 1.1$ ).

In Figure 2 we numerically compare the two QSS approximations in the  $S$ – $C$  and the  $\bar{S}$ – $C$  phase planes. The solutions of  $dC/dt = 0$ , given by equation (6) and the valid root of equation (15), are shown as light lines in Figures 2*a* and *b*, respectively, for different values of  $E_0$ . The heavy lines represent exact solution trajectories for the corresponding values of  $E_0$ , with initial conditions  $C(0) = 0$  and  $S(0) = \bar{S}(0) = 0.1$ . An important assumption for the QSS approximation is that the change in the substrate during the fast transient is small, *i.e.* the initial portion of the trajectory should be nearly vertical in the phase plane.

On the basis of Figure 1 we expect for  $k_2 = 0.1$  that the standard QSSA will become invalid by increasing  $E_0$  while the tQSSA will remain unaffected. This is confirmed by Figures 2*a* and *b*. For  $E_0 = 0.01$  the trajectory starts out rapidly and vertically and then sharply turns left to slowly follow the QSS solution in both Figures 2*a* and *b*.



For  $E_0 = 1$  the trajectory of the standard model in Figure 2a depicts strong and rapid depletion of substrate before the relatively slow QSS solution is attained. This is not the case in Figure 2b, with the change of variables, even if  $E_0 = 100$ . Thus the tQSSA is valid, where the classical QSSA fails.

For  $k_2 = 10$  the expectation is more challenging. The standard QSSA should simply lose validity by increasing  $E_0$ . The tQSSA however should become worse at intermediate values of  $E_0$  but become accurate again at high values (by condition (24)). This is confirmed in Figures 2c and d. As expected the behaviour of the standard QSSA model in Figure 2c is comparable to that in Figure 2a. Moreover, the trajectories of the tQSSA model indeed show the strongest substrate depletion when  $E_0 = 1$ . Additionally, when  $E_0 = 1$  we see that after the fast transient the trajectory fails to approach the tQSS approximation. Remember that condition (23) pertains to both the difference in time scales and to the depletion of substrate.

## The reverse QSSA

There is an alternative approach to simplifying the governing equations (3), by assuming that  $dS/dt = 0$  rather than  $dC/dt = 0$ . This approach, termed the reverse QSSA (rQSSA for short), has been outlined by Segel & Slemrod [192]. Like the tQSSA, the rQSSA is valid when  $E_0$  is “large,” in contrast to the validity condition “ $E_0$  small” for the classical QSSA. In the Appendix we show that in the parameter range where the rQSSA is valid, our new and simpler tQSSA is valid also, and that both approximations give similar results.

## The replication model

Initial conditions with large  $E_0$  are typical of the *replication scheme* (2). The differential equations corresponding to scheme (2) are

$$\frac{dS}{dt} = -k_1(E_0 - C)S + (k_{-1} + 2k_2)C, \quad (26a)$$

$$\frac{dC}{dt} = k_1[(E_0 - C)S - K_m C], \quad (26b)$$

where equation (26b) is identical to equation (3b), and the conservation law and initial conditions are identical to equations (5a–d).

The standard QSSA approach for this replication model proceeds analogously to the analysis of equations (6–11). Thus, setting  $dC/dt = 0$  in equation (26b), we obtain

equation (6), which upon substitution into equation (26a) gives

$$\frac{dS}{dt} = k_2 C, \quad (27a)$$

*i.e.*

$$\frac{dS}{dt} = \frac{k_2 E_0 S}{K_m + S}. \quad (27b)$$

$S(0) = S_0$  is assumed as an “initial condition.” Note that in equation (27b)  $S$  increases by replication, in contrast to equation (3a) where  $S$  decreases by transformation into product.

Because the differential equations of the complex, *i.e.* equations (26b) and (3b) are the same for the standard and replication models, the fast time scale  $t_C$  is also the same and is hence given by equation (8). Upon contemplating the slow time scale  $t_S$  we observe that equation (27b) is identical to equation (7b) except for a difference in sign. However, we cannot employ the characterization (9a) for  $t_S$  because substrate now increases, *i.e.* the maximum change of substrate is not defined. Instead we propose a slightly modified estimate of  $t_S$ : we calculate the relative change in substrate, *i.e.*  $(dS/dt)/S$ , right after the fast transient, *i.e.* when  $S \approx S_0$ . Thus, we consider equation (27) for  $S(0) = S_0$ , divide by  $S_0$ , and take the inverse to obtain  $t_S$  given by equation (9). Note that this modified estimate also applies to the product model of equations (3a–b), and that both approaches give the same estimate for the slow time scale.

For consistency we require that the substrate is hardly depleted during the fast transient. From equation (26a) the maximum depletion rate is  $k_1 E_0 S_0$  which maximally lasts  $t_C$  time units. This again yields condition (11). Thus, we find equations (10) and (11) as the two conditions for the QSSA. We conclude that there is no difference in the QSSA conditions for the *standard* approaches to the product and replication models.

### Replacing free by total substrate: the tQSSA

Defining  $\bar{S} \equiv S + C$  as in equation (12) we write for scheme (2)

$$\frac{d\bar{S}}{dt} = k_2 C, \quad (28a)$$

$$\frac{dC}{dt} = k_1 [(E_0 - C)(\bar{S} - C) - K_m C], \quad (28b)$$

where the equation for  $dC/dt$  is again identical to equation (13b) and the initial conditions are given by equations (14a–b).

Solving  $dC/dt = 0$  we find equation (15) and its Padé approximant equation (17). For calculating the fast time scale  $t_C$  we employ equation (20) to obtain equation (21). For

the slow time scale  $t_{\bar{S}}$  we consider  $d\bar{S}/dt$  right after the fast transient, *i.e.* we use equation (28a) with  $C$  approximated by equation (17), and  $\bar{S} = S_0$ , to obtain equation (22) and hence equation (23). As before, equation (23) also guarantees the approximate initial condition (14a).

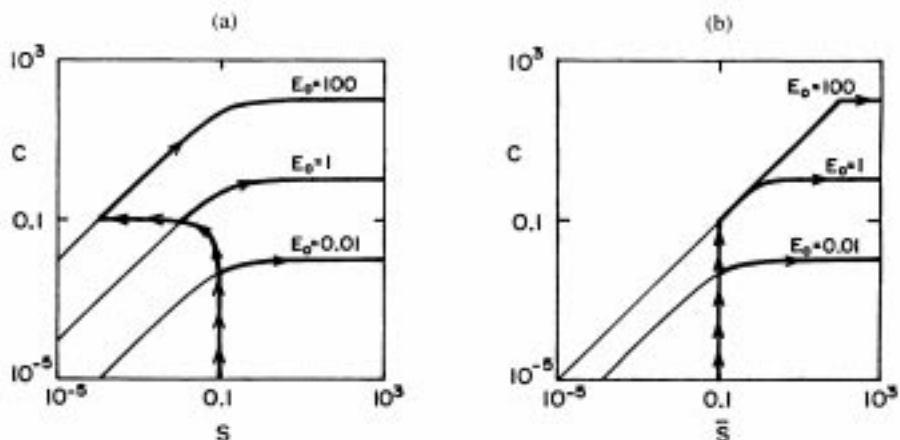


Figure 3. Similar to Figures 2a and b, but for the replication model (equations (26a–b)) with  $k_2 = 0.1$ . The light QSS curves are the same as in Figure 2 (but note different scales). Since now  $dS/dt > 0$ , these curves are traced “backwards.”

Our finding that the QSS approximation is extended by making a tQSSA is illustrated for the present example in Figure 3. The light lines depict the same QSS solutions as those in Figures 2a and b. The heavy lines again represent the exact trajectories for the same parameters and initial conditions. For  $E_0 = 0.01$  the trajectory has the required nearly vertical initial behaviour. For  $E_0 = 1$  and  $E_0 = 100$  the trajectories in Figure 3b have the required initial behaviour, whereas those in Figure 3a do not.

Figure 4 illustrates in a different way the advantage of the total substrate formulation in the replication model. We plot the free and total substrate concentrations,  $S$  and  $\bar{S}$ , as functions of time. Here F denotes the solution of the full model, *i.e.* equations (26a–b) or (28a–b), Q that of the QSSA model, *i.e.* equation (26a) with (6) or (28a) with the valid solution of equation (15), and P that of the Padé approximation, *i.e.* equation (28a) with (17). When  $E_0 = 0.01 < S_0 = 0.1$  the QSSA is valid in both models and all solutions are identical (not shown). If  $E_0 = 100 > S_0 = 0.1$  the two tQSS variants Q and P closely resemble the full solution F (Figure 4b). However, there is a large difference between Q and F in the classical model of Figure 4a: the “approximation” Q incorrectly shows a rapid growth in free substrate  $S$  while in fact  $S$  initially decays rapidly, owing to complex formation, before replication causes rapid growth of  $S$ . This defect is absent when we change variables to  $\bar{S}$  (see Figure 4b), for  $\bar{S}$  is not depleted by complex formation.

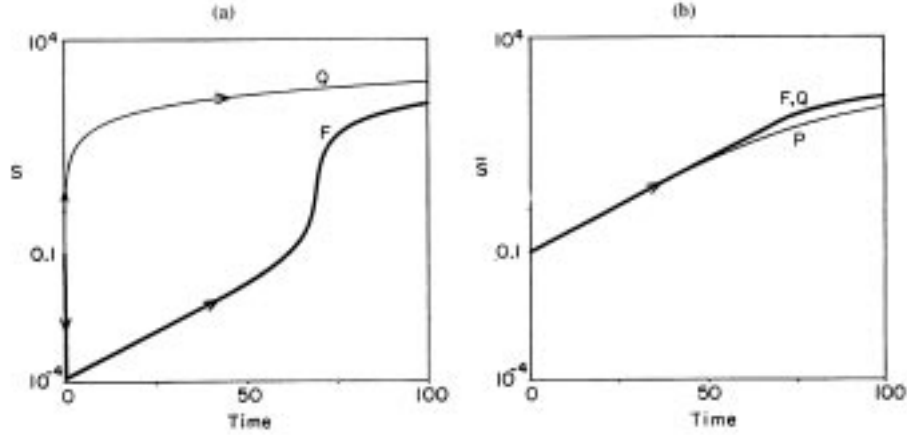


Figure 4. Time plots of free substrate concentration  $S$  and total substrate concentration  $\bar{S}$  for the replication model (equations (26a–b)), for high concentrations of enzyme. The solution of the full model is marked as F, that of the quasi-steady state model as Q, and that of the Padé approximation as P. Parameters are as in Figure 3. The initial conditions are  $C(0) = 0$ ,  $S(0) = \bar{S}(0) = 0.1$ , and  $E(0) = 100$ .

## T cell proliferation

De Boer & Perelson [63] modelled T cell growth on the basis of schemes similar to scheme (2). They derived the equivalent of equation (27), added a source and decay term, and proposed the following model of T cell growth, where “substrate” now means the free T cell density  $T$ :

$$\frac{dT}{dt} = \sigma + T \left( \frac{\rho A}{K + T} - 1 \right). \quad (29)$$

Here  $A$  is the concentration of sites presenting antigen and  $\sigma$  is the source of naive T cells from the thymus. Equation (29) naturally implements competition between T cells for seeing antigen. The interesting implications of this model are discussed by De Boer & Perelson [63]. The main problem with this model is that it has an unbounded *per capita* rate of T cell growth. The growth rate of a T cell population increases without bound as  $A \rightarrow \infty$ . We are now able to solve this problem by changing variables to total T cells,  $\bar{T} \equiv T + C$ , and using equations (17) and (28a–b) to write

$$\frac{d\bar{T}}{dt} = \sigma + \bar{T} \left( \frac{\rho A}{K + \bar{T} + A} - 1 \right). \quad (30)$$

Here  $\rho$  represents the maximum proliferation rate for an individual T cell (which is formally achieved when  $A \rightarrow \infty$ ). This model is much more realistic and also accounts for competition between T cells for seeing antigen.

A disadvantage of introducing  $\bar{T}$  is the quadratic equation one has to solve (*i.e.* equation (15)), which, for instance, prevents one from repeating the analysis of De Boer & Perelson [63] for  $n > 1$  T cell populations seeing  $m > 1$  different antigens. De Boer & Perelson [64] do derive a model for  $n$  T cell populations interacting with one antigen.

## Predator–prey interactions

The Lotka–Volterra model for a predator species feeding upon a prey species is classical in ecology. A general model for the interaction between a prey  $X$  and predator  $Y$  is

$$\frac{dX}{dt} = X(1 - X) - af(X, Y), \quad (31a)$$

$$\frac{dY}{dt} = abf(X, Y) - cY, \quad (31b)$$

where

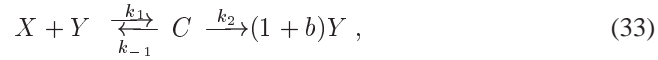
$$f(X, Y) = XY, \quad (32a)$$

or

$$f(X, Y) = \frac{XY}{K + X}. \quad (32b)$$

Equation (32b) is the Holling type II response. Since in equation (32b)  $f(X, Y) \propto Y$  when  $X \gg K$ , the parameter  $a$  is interpreted as the maximum number of prey eaten per predator per unit time.

Employing the approach outlined above we can formally derive a generalized interaction term  $F(X, Y)$  from the scheme



where  $b$  is the growth rate of the predator due to eating prey. Changing variables to total prey  $\bar{X} \equiv C + X$  and total predators  $\bar{Y} \equiv C + Y$  and making a QSSA for  $C$ , we obtain

$$\frac{dC}{dt} = k_1[(\bar{X} - C)(\bar{Y} - C) - K_m C] = 0, \quad (34a)$$

or

$$C \approx \frac{\bar{X}\bar{Y}}{K_m + \bar{X} + \bar{Y}}. \quad (34b)$$

In addition

$$\frac{d\bar{X}}{dt} = -k_2 C \quad (35a)$$

and

$$\frac{d\bar{Y}}{dt} = bk_2C. \quad (35b)$$

Writing  $k_2 = a$  and  $K_m = K$  we thus obtain

$$F(\bar{X}, \bar{Y}) = \frac{\bar{X}\bar{Y}}{K + \bar{X} + \bar{Y}} \quad (36)$$

as a general interaction function. Because this saturates as a function of  $\bar{X}$ , the parameter  $a$  in equations (31a–b) has the same interpretation as it has in the Holling type II response.

Having a general function, we observe that the Holling type II response is retrieved when we assume that there is an excess of prey, *i.e.*  $\bar{X} \gg \bar{Y}$ , so that from equation (34b)  $\bar{X} \gg C$ . Equation (34a) then simplifies into

$$\frac{dC}{dt} = k_1[\bar{X}(\bar{Y} - C) - K_m C] = 0 \quad \text{giving} \quad F(\bar{X}, \bar{Y}) = \frac{\bar{X}\bar{Y}}{K + \bar{X}}. \quad (37)$$

Similarly, assuming an excess of predators, *i.e.*  $\bar{Y} \simeq Y$ , equation (34a) simplifies into

$$\frac{dC}{dt} = k_1[\bar{Y}(\bar{X} - C) - K_m C] = 0 \quad \text{giving} \quad F(\bar{X}, \bar{Y}) = \frac{\bar{X}\bar{Y}}{K + \bar{Y}}, \quad (38)$$

which allows for interference between predators. The parameter  $a$  however loses the above interpretation.

Our general function given by equation (36) has been proposed previously on intuitive grounds by DeAngelis *et al.* [67] and Beddington [20]. The fact that this function can now be formally derived supports its usage in ecological models, and provides clues and/or precautions for how to generalize equation (36) for a system with several predator and prey species (see [64]). Functions like this have also been proposed in the context of ratio-dependent predator–prey interactions [9]. We think equation (36) is appropriate in this context because for  $X \gg K$  it resembles the Holling function, with its maximum rate of predation per predator, whereas for  $X \ll Y$  it allows for the interference between predators that is characteristic of models with ratio-dependent predation.

## Summary and discussion

Rather remarkably, there is still more to say about the standard enzyme–substrate–complex scheme (1a), with its mathematical formulation given by equations (3)–(5). In the

classical approach, the concentrations of free substrate  $S$  and the complex  $C$  are taken as dependent variables. The quasi-steady state assumption (QSSA)  $dC/dt = 0$  is made, resulting in equation (6) for  $C$  as a function of  $S$  and hence differential equation (7b) for  $dS/dt$ . It is assumed that little substrate is consumed during the initial transient period before the QSSA is valid, so that  $S(0) = S_0$  is taken as the initial condition for equation (7b). The validity of this procedure is assured if condition (11) holds.

We have explored a different approach, where the total substrate concentration  $\bar{S}$  is employed instead of  $S$ . Again the assumption  $dC/dt = 0$  is made, but now this results in the quadratic equation (15) for  $C$  as a function of  $\bar{S}$ . We show that equation (17) offers an accurate Padé approximation for equation (15), which provides an explicit formula for  $C$ . An equation for  $d\bar{S}/dt$  can now be written, which is solved subject to the initial condition  $\bar{S}(0) = S_0$ . The validity of this total QSSA (tQSSA) is assured by condition (23).

By changing variables from free substrate  $S$  to total substrate  $\bar{S}$  we have enlarged the domain of parameters for which it is permissible to employ the classical QSSA,  $dC/dt = 0$ . It might be objected that this is getting something for nothing, for how can the same assumption be rendered more acceptable merely by a simple change of variables? However, recall (see [189]) that the essential reason why the QSSA holds is that the QSS variable (here  $C$ ) has a fast intrinsic rate of change compared to the “non-QSS variable” (here  $S$ ). For the parameter range of interest, our new “non-QSS variable”  $\bar{S}$ , the total substrate concentration, changes very much more slowly than  $S$ , and hence our change of variable should indeed lead to an improved approximation.

The classical QSSA loses its validity when condition (11) fails, *i.e.* when there is little substrate compared to enzyme. The allegedly slow (substrate) variable then becomes fast due to rapid formation of complex. This effect, which is particularly important during the initial transient, is evaded by the change of variables to total substrate, because total substrate can only change by the formation of product. This explains why  $k_2$  appears in conditions (23) and (24).

When checking the validity of the classical QSSA, for example, we ascertained that the phase plane curve  $C = C(S)$  of equation (6) was indeed approached by the numerical solution of the governing equations. This check also indicates that the graphs for  $S(t)$  and  $C(t)$  that are derived from the QSSA will be close to the correct values. The reason is that if indeed equation (6) holds, then equation (7b) is a good approximation to the true equation for  $dS/dt$ . Hence the solution of equation (7b) should yield a good approximation, provided that the basic problem is not ill conditioned (so that a small error in the problem can lead to a large error in the solution) and provided that integration has not been carried out so long that even large errors have had a chance to accumulate.

For definiteness, in this paragraph we continue to discuss general matters in the framework of the classical QSSA. We have stressed that justification of the QSSA requires demonstrating two things, for the parameter domain in question. These are (i) that after a fast transient, one can approximate the  $dC/dt$  equation by regarding it at steady

state, yielding  $C = C(S)$ , and (ii) that the true initial condition  $S = S_0$  remains approximately true after the transient. The path to demonstrating (ii) was transparent — estimate the change in  $S$  during the transient. It is not clear, however, that our checking whether  $t_C \ll t_S$  is the right way to demonstrate (i). (Although it is clear that consistency demands that the duration of the fast transient is relatively brief.) It appears that the best way to show that  $t_C \ll t_S$  ensures that  $C = C(S)$  is to use  $t_C$  and  $t_S$  in a formal adoption of suitable scaled variables, as described by Segel & Slemrod [192]. These authors show that  $C = C(S)$  emerges from a singular perturbation analysis where the small parameter is the ratio  $t_C/t_S$ .

The change of variables into total substrate may give rise to complications in situations where the substrate also reacts with other variables. In the standard model one may assume that free substrate reacts with another variable but substrate in complex does not. Making the change of variables one typically writes that the total substrate variable, and hence both free substrate and complex, reacts with other variables. In the context of immunology, for an example of a T cell population interacting with both antigen and another (regulator) T cell population, see Borghans & De Boer [29].

When the work reported here was essentially completed, we learned that there were antecedents for part of it. With precedent from the careful studies of Straus & Goldstein [206] and Goldstein [89] on enzyme–substrate–inhibitor systems, Reiner [175], Cha & Cha [48] and Cha [47] used total substrate concentration in pursuing the implications of the QSS assumption  $dC/dt = 0$ . They thus employed equation (15) to determine  $C$  as a function of  $\bar{S}$ . They discussed different approximations of equation (15) in different parameter domains, but they never challenged the basic assumption  $dC/dt = 0$ . That this assumption could well be inappropriate was recognized by Lim [121], who illustrated his assertion by a numerical example.

Novel delineation of the domains of validity of the various approximations, our central goal. Perhaps it should go without saying that the approach taken here can also be applied, at least in principal, to the many other kinetic equations where some type of QSSA could be appropriate.

## Appendix: the reverse QSSA

We here present the reverse QSSA (rQSSA for short) of Segel & Slemrod [192] in a somewhat altered and extended form, since we wish to compare its results with those obtained above. Upon setting  $dS/dt = 0$  in equation (3a) we obtain

$$S = \frac{KC}{E_0 - C}, \quad (39)$$



where

$$K = k_{-1}/k_1. \quad (40)$$

Substitution of equation (39) into equation (3b) yields

$$\frac{dC}{dt} = -k_2 C. \quad (41)$$

(Another way to obtain equation (41) is to add equation (3a) to (3b) after setting  $dS/dt = 0$ .) The complex concentration  $C$  typically decreases substantially during the transient, but nonetheless an appropriate initial condition for equation (41) can be derived [192].

A necessary condition for the validity of the rQSSA is that the time scale  $t_C$  for post-transient changes in  $C$  is long compared to the time scale  $t_S$  for the rapid transient changes in  $S$  that occur before equation (39) is satisfied. From equation (41),  $t_C = k_2^{-1}$ . To estimate  $t_S$  we begin by employing equation (12) to replace  $S$  by  $\bar{S} - C$  in equation (39). Rearranging the resulting equation we obtain

$$C^2 - (E_0 + \bar{S} + K)C + E_0\bar{S} = 0. \quad (42)$$

Equation (42) differs from equation (15) only in that  $K$  appears in equation (42) where  $K_m$  appears in equation (15). Given that  $\bar{S}$  remains close to  $S_0$  during the transient, we can replace  $K_m$  by  $K$  in the Padé formula (17) to estimate that at the end of the transient,  $C$  has the value  $\tilde{C}$ , where

$$\tilde{C} = \frac{E_0 S_0}{E_0 + K + S_0}. \quad (43)$$

$C(0) = \tilde{C}$  is the appropriate “initial condition” for equation (41).

Replacing  $C$  by  $\tilde{C}$  in equation (3a) yields

$$\frac{dS}{dt} = -k_1(E_0 - \tilde{C})S + k_{-1}\tilde{C}. \quad (44)$$

It follows that  $[k_1(E_0 - \tilde{C})]^{-1}$  provides an estimate of  $t_S$ , i.e.

$$t_S \approx \frac{K + E_0 + S_0}{k_1 E_0 (K + E_0)}. \quad (45)$$

This estimate is conservative ( $t_S$  is slightly overestimated). From the condition  $t_S \ll t_C$ , we thereby obtain the following conservative estimate for the parameter domain wherein the rQSSA is valid:

$$\frac{k_2}{k_1} \ll \frac{(K + E_0)E_0}{K + E_0 + S_0}. \quad (46)$$

By comparison, condition (23) for the validity of the “total” or tQSSA can be written

$$\frac{k_2}{k_1} \ll \frac{(K_m + E_0 + S_0)^2}{E_0}. \quad (47)$$

The tQSSA is valid in a strictly larger parameter domain than the rQSSA because

$$\frac{(K_m + E_0 + S_0)^2}{E_0} > \frac{(K + E_0 + S_0)^2}{E_0} > \frac{(K + E_0)E_0}{K + E_0 + S_0}. \quad (48)$$

When both the tQSSA and the rQSSA are valid, that is when condition (46) holds, consistency demands that both approximations be virtually identical. Comparing equations (42) and (15), we see that this will be the case if

$$E_0 + \bar{S} + K_m \approx E_0 + \bar{S} + K, \quad (49)$$

or

$$k_1 E_0 + k_1 \bar{S} + k_2 + k_{-1} \approx k_1 E_0 + k_1 \bar{S} + k_{-1}. \quad (50)$$

However, condition (50) indeed holds, since condition (46) implies that  $k_2 \ll k_1 E_0$ . Thus the tQSSA is to be preferred because it has a wider range of validity than the rQSSA and it does not require special derivation of a post-transient “initial condition.”

# 7

## **Competition for antigenic sites during T cell proliferation: a mathematical interpretation of *in vitro* data**

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## Abstract

By fitting different mathematical T cell proliferation functions to *in vitro* T cell proliferation data, we studied T cell competition for stimulatory signals. In our lymphocyte proliferation assays both the antigen availability and the concentration of T cells were varied. We show that proliferation functions involving T cell competition describe the data significantly better than classical proliferation functions without competition, thus providing direct evidence for T cell competition *in vitro*. Our mathematical approach allowed us to study the nature of T cell competition by comparing different proliferation functions involving (i) direct inhibitory T–T interactions, (ii) antigen-specific resource competition, or (iii) resource competition for nonspecific factors such as growth factors, and access to the surface of antigen-presenting cells (APCs). We show that resource competition is an essential ingredient of T cell proliferation. To discriminate between antigen-specific and nonspecific resource competition, the antigen availability was varied in two manners. In a first approach we varied the concentration of APCs, displaying equal ligand densities; in a second approach we varied the antigen density on the surface of the APCs, while keeping the APC concentration constant. We found that both resource competition functions described the data equally well when the antigen availability was increased by adding APCs. When the APC concentration was kept constant, the nonspecific resource competition function yielded the best description of the data. Our interpretation is that T cells were competing for “antigenic sites” on the APCs.

## Introduction

Competition between lymphocytes for stimulatory and survival signals is thought to play a pivotal role in the homeostatic control of the immune system. The steady-state population sizes of naive and memory T cell compartments [214–216], and of resting B cell and activated IgM-secreting B cell compartments [2, 3] are all independently regulated by cellular competition within each compartment. Due to competition between lymphocytes, cellular death rates and/or renewal rates are density-dependent functions of the peripheral population sizes [80, 82, 216]. By such density-dependent mechanisms homeostasis is established.

There is a qualitative difference between the regulation of total T and B cell numbers. Although it has been shown that part of the B cell repertoire is maintained by competitive renewal in the periphery [2], B cells predominantly compete for survival signals [82]. Memory T cells, however, mainly compete for stimulatory signals [80], affecting proliferation rates at high T cell concentrations. Here we study T cell competition and focus on the effect of high concentrations of T cells with the same specificity on the rate of T cell proliferation.

The nature of the signals for which T cells are competing remains elusive. Previous

experiments have suggested that T cell competition is antigen-specific. CD8<sup>+</sup> T cell competition was studied *in vivo* by reconstituting lethally irradiated mice with mixtures of precursor bone marrow cells from normal nontransgenic and T cell receptor (TCR)-transgenic mice [80]. It was shown that the proliferative capacity of the TCR-transgenic cells was diminished in the presence of other T cells, indicating that competition between T cells occurred. Moreover, nontransgenic cells appeared to have a selective advantage over TCR-transgenic cells in seeding the peripheral lymphoid tissue, suggesting that cells were competing for antigens.

Lymphocyte competition may, however, also act at a more global level, if lymphocytes compete for nonspecific factors such as growth factors, nutrients or access to the surface of APCs. The fact that transgenic mice attain total peripheral lymphocyte numbers similar to those in normal mice has been interpreted as evidence for a global homeostatic control, acting independently of cell specificity [81]. This argument was weakened by a mathematical model which showed that such equal total lymphocyte numbers could also be obtained when only an antigen-specific homeostatic control was taken into account [65]. The experimental data [80, 81] thus fail to give a decisive answer about the nature of the factors controlling immune homeostasis. The advantage of an antigen-specific homeostatic control would be that the diversity of the immune system can be maintained. If all clonotypes were to compete for the same resource — *e.g.* a growth factor — the clonotype responding most vigorously would outcompete all other clonotypes [63].

In this chapter we studied T cell proliferation, and in particular the nature of T cell competition, by fitting several mathematical proliferation functions to data from *in vitro* lymphocyte proliferation assays. Proliferation was measured both as a function of the antigen concentration and as a function of the number of T cells competing for antigen. The aim of this study was twofold: on the one hand to provide insights into the relative importance of inhibitory T–T interactions, antigen-specific resource competition, and nonspecific resource competition in T cell proliferation, by mathematical analysis of *in vitro* data; on the other hand, to provide an experimental validation of several T cell proliferation functions that are frequently used in theoretical immunology. Briefly, our analysis shows that T cell proliferation functions allowing for T cell competition describe the experimental data significantly better than conventional noncompetitive saturation functions. This demonstrates that T cell competition plays a role *in vitro*. We show that most of the competition in our assays can be attributed to competition for antigenic sites on APCs.

## Materials and methods

### T cells and antigens

The generation and maintenance of the CD4<sup>+</sup> Z1a T cell clone have been described previously [22]. Briefly, the Z1a T cell clone was derived from the draining lymph nodes of a Lewis rat immunized in the hind footpads with guinea pig myelin basic protein (MBP) in complete Freund's adjuvant. T cell clone Z1a is reactive with the 72–85 amino acid sequence of MBP and with peptide 72–85<sub>S79A</sub>, an analogue of the native peptide which has a higher MHC class II RT1.B<sup>L</sup> binding affinity [226]. T cells were cyclically restimulated *in vitro* for 3 or 4 days in the presence of irradiated (3000 rads) thymocytes as APCs and 10 µg/ml MBP, and propagated for 6 or 7 days. Cells were restimulated in Iscove's modified Dulbecco's medium (Gibco), supplemented with 2% Lewis rat serum, 2mM *L*-glutamine, (50 µM) 2-mercaptoethanol, and antibiotics. Propagation was performed in Iscove's modified Dulbecco's medium, supplemented with 2mM *L*-glutamine, (50 µM) 2-mercaptoethanol, antibiotics, 10% fetal calf serum (FCS), 10% EL-4 supernatant (IL-2 source), and 1% nonessential amino acids. All experiments were also performed with the Lewis rat CD4<sup>+</sup> T cell clone A2b, specific for the 176–190 amino acid sequence of mycobacterial heat shock protein HSP65 [8, 97], yielding similar results (data not shown).

### T cell proliferation

Proliferative responses of T cells were measured in triplicate cultures in flat-bottom microtiter plates (Costar). T cells were cultured at different concentrations in 0.2 ml Iscove's modified Dulbecco's medium supplemented with 5% FCS, 2mM *L*-glutamine, (50 µM) 2-mercaptoethanol, and antibiotics in the presence of irradiated (3000 rads) thymocytes as APCs. To exclude any effects of free antigen or T–T cell presentation [210], T cells were incubated with APCs that had been prepulsed with peptide. APCs were prepulsed ( $6 \times 10^7$  cells per ml) with MBP 72–85<sub>S79A</sub> (or HSP65 176–190, data not shown) for 1.5 hours at 37 °C (5% CO<sub>2</sub>) and thoroughly washed. In our first approach, APCs were prepulsed with a standard peptide concentration (100 µg/ml), after which T cells were incubated with different concentrations of APCs (varying from  $0.5 \times 10^6$  to  $2.5 \times 10^6$  cells per well). In the second approach, APCs were prepulsed with different concentrations of peptide (varying from 1 to 500 µg/ml), after which T cells were incubated with a standard concentration of APCs ( $1 \times 10^6$  cells per well). T cell concentrations varied from  $0.5 \times 10^4$  to  $32 \times 10^4$  cells per well. Total T cell proliferation was measured at 24 hours by addition of [<sup>3</sup>H]-thymidine during the last 16 hours of a 24 hours culture period. Cells were harvested on fibreglass filters, and [<sup>3</sup>H]-thymidine incorporation was measured by liquid scintillation counting.

## Statistical procedures

The optimal fits of the mathematical functions to the data were determined by using a generalized Gauss–Newton method to minimize the sum of the squared residuals (SSR) between the logarithms of the experimental and theoretical data. The logarithmic transformation was made because the experimental errors were likely to be proportional to the [<sup>3</sup>H]-thymidine incorporation levels measured. To ascertain that the minima found were not reflecting local minima, the optimization procedure was repeated for various initial conditions. All conditions tested gave rise to the same minimal SSR and parameter values.

## T cell proliferation functions

### Without competition

In theoretical studies of the immune system, it is common practice to describe T cell proliferation as a linear function of the concentration of antigen-specific T cells  $T$ , saturating over the concentration of antigen-presenting sites on APCs  $A$ :

$$T^* = \rho T \frac{A}{A + K}. \quad (1)$$

Here,  $T^*$  is a measure of total T cell proliferation, *i.e.* the amount of [<sup>3</sup>H]-thymidine incorporation in our experimental assays. The parameter  $\rho$  represents the maximum [<sup>3</sup>H]-thymidine incorporation of T cells, and  $K$  is a saturation constant giving the concentration of antigenic sites at which the rate of T cell proliferation is half-maximal. This saturation function describes the typical picture that is observed *in vitro*: total T cell proliferation increases with the antigen concentration until a certain plateau level is reached. In some experiments, T cell proliferation decreases at very high antigen concentrations, leading to log bell-shaped proliferation curves [131]. Here we focused only on the first part of the curves, where T cell proliferation increases when the antigen concentration increases.

According to the conventional proliferation function of equation (1), doubling the number of T cells doubles total [<sup>3</sup>H]-thymidine incorporation, regardless of the antigen availability. When antigen becomes limiting, this function may behave unrealistically, as T cells are expected to compete for the limiting antigenic resource. Thus, a T cell competition term may be an essential ingredient of T cell proliferation functions.

## Inhibitory T–T interactions

Several mechanisms have been described by which T cells may directly influence each others' proliferation/survival. By cytokine secretion [90] or consumption [91, 124], T cells may inhibit cell division of other T cells in the local environment. Alternatively, T cells may present antigens to other T cells, generally inducing the responding T cells to become anergic [210]. If T lymphocytes indeed directly hinder each other by means of inhibitory T–T interactions, total T cell proliferation can be described by:

$$T^* = \rho T \frac{A}{A + K} - \varepsilon T^2, \quad (2)$$

where T cell competition is modelled by the  $\varepsilon T^2$  term (see [33] for an application). According to equation (2), a T cell approaches its maximum proliferation rate  $\rho$  when the antigen availability is large and the T cell population is small; the proliferation per T cell decreases when the total concentration of T cells increases.

## Antigen-specific resource competition

We have previously proposed, and applied [29], an alternative proliferation function, based on the conjecture that T cells inhibit each other indirectly by competing for a limiting antigenic resource [32, 64]. Such a function can be derived from the interactions between free T cells  $T_f$  and free antigenic sites on APCs  $A_f$ . When a T cell binds to a free antigenic site on an APC, it forms a complex  $C$  which may either dissociate or lead to T cell proliferation. Thus, a cellular immune response can be represented by the following interaction scheme:



where the constants  $k_1$  and  $k_{-1}$  are reaction rates, and new T cells are formed by proliferation proportionally to the number of T cell–antigen complexes  $C$ . In the Appendix we show that this scheme yields the following T cell proliferation function:

$$T^* = \rho T \frac{A}{A + cT + K}, \quad (4)$$

where  $A$  is the total concentration of antigenic sites (i.e.  $A = A_f + C$ ), and  $T$  is the total concentration of antigen-specific T cells (i.e.  $T = T_f + C$ ). In equation (4),  $c$  reflects the degree of T cell competition for antigen binding. If  $c = 0$  this function amounts to the conventional saturation function of equation (1). A  $c$  value larger than zero would indicate that T cells are indeed competing for their antigenic resource. Competition between T cells thus results naturally from the decreasing antigen availability due to T cell–antigen complex formation.



## Nonspecific resource competition

T cells may also compete for nonspecific resources, such as growth factors, nutrients and/or access to the surface of APCs. To account for such an antigen-independent form of competition, we propose a third T cell proliferation function. Assuming that competition for nonspecific resources affects the maximum proliferation rate of T cells, total T cell proliferation can be described by:

$$T^* = \rho \frac{T}{1 + sT} \frac{A}{A + K}, \quad (5)$$

where  $s$  reflects the degree of T cell competition. Again, the proliferation per T cell decreases when the total concentration of T cells increases. Unlike the antigen-specific competition term of equation (4), the nonspecific competition term of equation (5) involves all T cell clones competing for the same nonspecific resource, e.g. all clones recognizing their antigen on the same APC.

## Results and discussion

The effect of T cell competition on proliferation was studied *in vitro*, by performing lymphocyte proliferation assays with the encephalitogenic CD4<sup>+</sup> T cell clone Z1a. In the assays, both the concentration of T cells and the concentration of antigen were varied. In a first approach, APCs were prepulsed with a standard concentration of peptide, after which increasing concentrations of prepulsed APCs, displaying the same ligand densities, were incubated with T cells.

To minimize any changes in the numbers of T cells in the wells during the experiment, proliferation had to be measured as early as possible. In a pilot study, [<sup>3</sup>H]-thymidine incorporation for several combinations of antigen and T cell concentrations was measured at different time points (*i.e.* 17, 24, 40, 65, and 88 hours) after the start of the incubation period. At all time points T cell proliferation could be detected (data not shown). At the earliest time point (*i.e.* 17 hours), the dose–response curves of several T cell concentrations did not yet saturate as a function of the antigen concentration. From 24 hours onwards, the typically observed picture of proliferation saturating as a function of the antigen concentration was found (data not shown). Therefore, in all further experiments total T cell proliferation was measured by the [<sup>3</sup>H]-thymidine incorporation 24 hours after incubation.

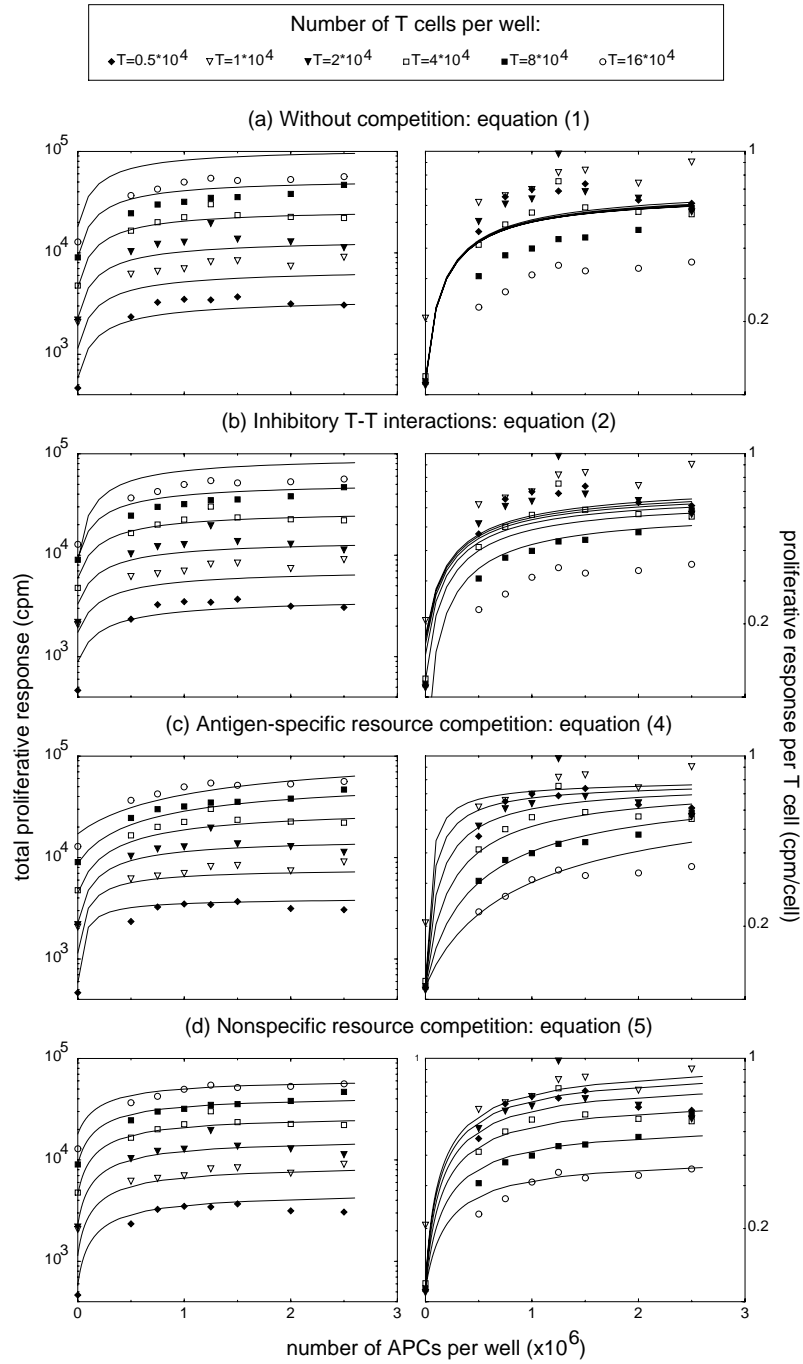


Figure 1. Proliferative responses of different concentrations of Z1a T cells in response to different concentrations of equally prepulsed APCs. Graphs compare experimental results (in symbols) and best theoretical fits (curves). The experimental data were fitted to (a) the conventional saturation function without T cell competition (equation (1)), (b) the proliferation function involving inhibitory T–T interactions (equation (2)), (c) the antigen-specific resource competition function (equation (4)), and (d) the nonspecific resource competition function (equation (5)), respectively. In the left-hand panels the data are expressed as total proliferative responses, whereas in the right-hand panels the same data are expressed as proliferative responses per T cell. Parameters of the theoretical curves are listed in Table 1.

To study which of the T cell proliferation functions derived above could give the best description of the experimental data, the different functions were fitted to the results of the proliferation assays. For each of the proliferation functions the set of parameters giving the best fit to the data was computed by minimization of the SSR between the experimental data and the function studied.

Because the experimental data included background [<sup>3</sup>H]-thymidine incorporation, the proliferation functions first had to be extended with a term accounting for background proliferation. Background proliferation increased with the concentration of T cells. This proliferation was probably due to prior T cell stimulation, since the Z1a and A2b clones were maintained by a weekly phase of restimulation and expansion. In the absence of T cells, [<sup>3</sup>H]-thymidine incorporation was low (< 200 cpm). Assuming that background T cell proliferation was indeed due to prior T cell stimulation, we modelled it as a term independent of T cell–antigen complex formation. Thus, in the fitting procedure all functions described above were extended by adding the term  $\beta = b_T T + b_A A + b$ , accounting for background [<sup>3</sup>H]-thymidine incorporation due to T cells, background incorporation due to APCs, and background incorporation in the absence of both T cells and APCs, respectively.

Figure 1 summarizes the results. All panels represent the same set of experimental data, denoted by the symbols. The left-hand panels show the total T cell proliferative responses, while the right-hand panels show the same data expressed as the proliferative responses *per T cell*. From the left-hand panels of Figure 1 it can be seen that total T cell proliferation increased both with the concentration of T cells and with the APC concentration. If competition for T cell proliferation occurs, one would expect the proliferative responses *per T cell* to decrease when the T cell concentration increases. Indeed, the right-hand panels of Figure 1 show that T cells at high T cell concentrations had a lower proliferation rate per T cell than cells at low T cell concentrations. Thus T cell competition played a role in our assays.

Exp. Approach	Function	$\rho$	$K_1$	$c_1$	$s$	$\varepsilon$	$b_T$	$b_A$	$b$
1	No competition (eq. 1)	0.56 (0.10)	$3.9 \times 10^5$ ( $2.5 \times 10^5$ )				0.11 (0.01)	$4.2 \times 10^{-5}$ ( $0.8 \times 10^{-5}$ )	18 (5.3)
	Inhib. T-T (eq. 2)	0.53 (0.09)	$4.4 \times 10^5$ ( $2.4 \times 10^5$ )			$7.8 \times 10^{-7}$ ( $1.6 \times 10^{-7}$ )	0.18 (0.02)	$4.3 \times 10^{-5}$ ( $0.7 \times 10^{-5}$ )	18 (4.5)
	Ag-spec. res. comp. (eq. 4)	0.65 (0.06)	$0.2 \times 10^5$ ( $1.1 \times 10^5$ )	15.0 (2.7)			0.11 (0.01)	$4.2 \times 10^{-5}$ ( $0.4 \times 10^{-5}$ )	18 (2.9)
	Nonsp. res. comp. (eq. 5)	0.86 (0.09)	$3.8 \times 10^5$ ( $1.4 \times 10^5$ )		$1.3 \times 10^{-5}$ ( $0.2 \times 10^{-5}$ )		0.11 (0.01)	$4.2 \times 10^{-5}$ ( $0.4 \times 10^{-5}$ )	18 (2.9)
			$\rho$	$K_2$	$c_2$	$s$	$\varepsilon$	$b_T$	
2	No competition (eq. 1)	0.18 (0.02)	8.9 (3.1)				0.05 (0.006)		67 (8.1)
	Inhib. T-T (eq. 2)	0.17 (0.02)	9.7 (3.2)			$1.1 \times 10^{-7}$ ( $0.3 \times 10^{-7}$ )	0.06 (0.007)		65 (7.3)
	Ag-spec. res. comp. (eq. 4)	0.21 (0.02)	4.5 (2.3)	$2.0 \times 10^{-4}$ ( $0.8 \times 10^{-4}$ )			0.05 (0.005)		65 (6.4)
	Nonsp. res. comp. (eq. 5)	0.29 (0.02)	9.0 (1.7)		$0.9 \times 10^{-5}$ ( $0.2 \times 10^{-5}$ )		0.05 (0.003)		65 (4.6)
			$\rho$	$K_2$	$c_2$	$s$	$\varepsilon$	$b_T$	

Table 1. Results of the curve-fitting procedure of the first and second experimental approaches. Parameters were estimated by minimizing the SSRs between the logarithms of the total responses and the proliferation functions. Parameters giving the best fit to the data are followed by the corresponding standard deviations in parentheses. Background proliferation was modelled by the term  $\beta = b_T T + b_A A + b$ . In the second experimental approach, in which the concentration of APCs was not varied, no discrimination could be made between background proliferation due to APCs and background proliferation in the absence of APCs. Therefore both sources of background [<sup>3</sup>H]-thymidine incorporation in the latter experiment were combined in the  $b$  term. Except for the  $K$  and  $c$  parameters, the parameter values of the two experimental approaches are of the same order of magnitude. The differences between the  $K$  and  $c$  parameters of the two approaches reflect the two different ways in which the antigen concentration was varied:  $K_1$  and  $c_1$  involve the number of antigenic sites per APC ( $n$ ), whereas  $K_2$  and  $c_2$  involve the number of antigenic sites established per  $\mu\text{g}$  peptide ( $m$ ). Indeed, the estimates of the  $K$  parameters and the  $c$  parameters differ by the same order of magnitude, *i.e.* about 5 orders of magnitude. The parameters  $\rho$  and  $b_T$  are given in cpm/(cells per ml),  $b_A$  in cpm/(sites per ml),  $K_1$  and  $K_2$  in sites per ml,  $c_1$  and  $c_2$  in sites per cell,  $\varepsilon$  in cpm/(cells per ml)<sup>2</sup>,  $s$  in 1/(cells per ml) and  $b$  in cpm.

The best theoretical fits between the total T cell proliferative responses and the proliferation functions derived above are denoted by the curves in the left-hand panels of Figure 1. It should be emphasised that in each panel all data were fitted simultaneously, explaining why the individual theoretical curves do not optimally fit the individual T cell concentration data sets. The parameter sets for which the optimal fits were obtained are given in Table 1. Although all four functions yielded a reasonable fit to the data, the right-hand panels of Figure 1 expose the shortcomings of both the conventional proliferation function without competition (Figure 1*a*), and the function involving inhibitory T–T interactions (Figure 1*b*). Both functions failed to account for the inhibitory effect of large T cell numbers on the proliferative response per T cell. Only the functions incorporating resource competition (either antigen-specific, Figure 1*c*, or nonspecific, Figure 1*d*) yielded good descriptions of the proliferative responses per T cell, indicating that T cells were competing indirectly, for shared resources.

Statistical analysis of the sums of the squared residuals (SSRs) of the different proliferation functions yielded that all proliferation functions involving T cell competition gave a significantly better fit to the data (F-test,  $p < 0.001$ ) than the conventional saturation function without competition (see Figure 2). In Figure 2 the antigen-specific resource competition of equation (4) is denoted by A, the nonspecific resource competition function of equation (5) by N, the inhibitory T–T interaction function of equation (2) is denoted by T, and the conventional saturation function of equation (1) by C. Solid arrows denote model extensions giving significantly better fits to the data, while dashed arrows denote extensions that did not lead to significantly better fits. It was of interest to determine whether extension of the competition functions with an extra competition term could significantly improve the fit to the experimental data. To this end, three new proliferation functions, each combining two of the competition terms described above (denoted by NA, AT, and NT) were fitted to the experimental data. Figure 2 shows that once the proliferation function involved resource competition (A or N), the fit to the experimental data could not be significantly improved by adding another competition term.

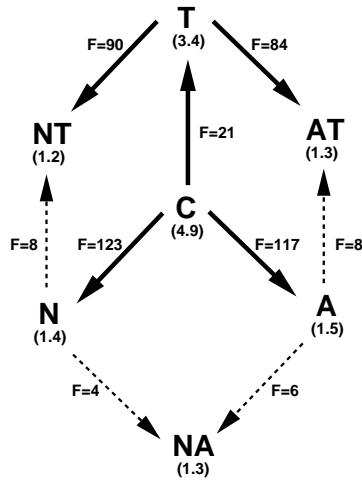
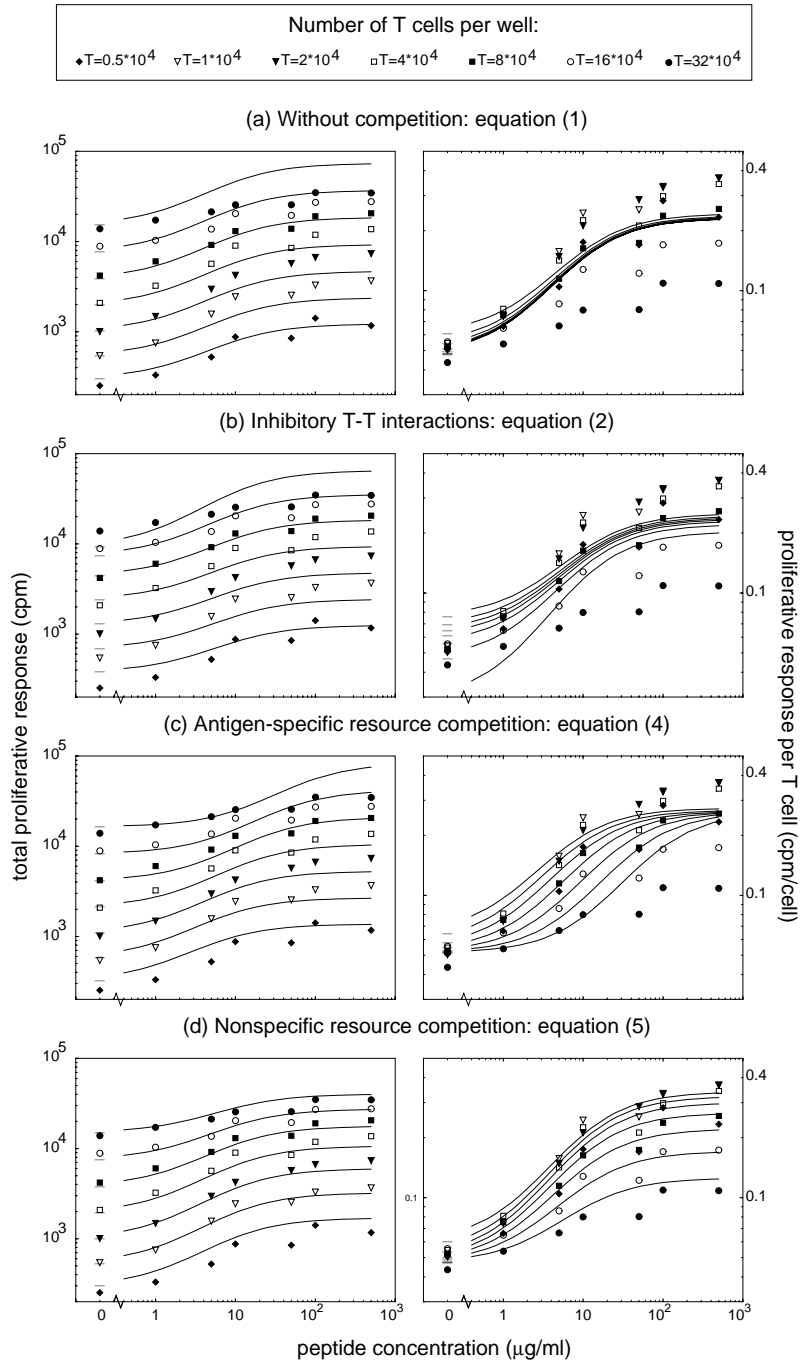


Figure 2. Statistical comparison of the SSRs of the different proliferation functions of the first experimental approach, with SSRs in parentheses. Arrows represent different model extensions and are accompanied by the corresponding F values. Solid arrows denote model extensions that significantly improved the fit to the experimental data (F-test,  $p < 0.001$ ); dashed arrows denote extensions that did not lead to significantly better fits ( $p > 0.001$ ). C represents the conventional saturation function without competition (equation (1)), T the inhibitory T–T interaction function (equation (2)), A the antigen-specific resource competition function (equation (4)), and N the nonspecific resource competition function (equation (5)). The proliferation function denoted by NA combines both forms of resource competition, AT combines antigen-specific competition and inhibitory T–T interactions, and NT combines nonspecific resource competition and inhibitory T–T interactions.

The proliferation function involving inhibitory T–T interactions (T), however, could be improved by incorporating either form of resource competition. Thus the SSR analysis demonstrates that T cell competition was mainly due to resource competition.

Figure 3. Proliferative responses of different concentrations of Z1a T cells in response to a standard concentration of APCs prepulsed with different concentrations of MBP 72–85<sub>S79A</sub>. For details see the legend of Figure 1.



To study the nature of the resources for which the T cells were competing, the analysis was repeated with a different experimental approach. APCs were incubated at a standard concentration with Z1a T cells, after prior prepulsing with increasing concentrations of peptide, leading to increasing ligand densities on the APCs. The results are summarized in Figure 3. Because of the wide range of antigen concentrations, the results of the second experimental approach were plotted on a logarithmic horizontal axis. This explains the sigmoid shape of the proliferation curves. The proliferation function involving non-specific resource competition (Figure 3d) gave the best description of the data. Both the standard saturation function without competition (Figure 3a) and the competition function involving inhibitory T-T interactions (Figure 3b) failed to describe the inhibitory effect of large T cell concentrations on the proliferation per T cell, demonstrating again that resource competition plays a role *in vitro*.

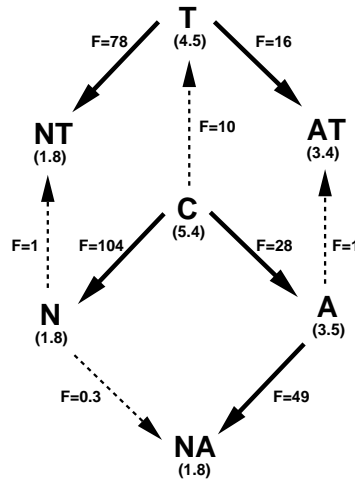


Figure 4. Statistical comparison of the SSRs of the different proliferation functions of the second experimental approach. For details see the legend of Figure 2.

The SSR analysis of the second experimental approach is summarized in Figure 4. Extending the conventional saturation function of equation (1) (C) with inhibitory T-T interactions (T) did not significantly improve the fit to the data, whereas extension with a resource competition term (A or N) again did. Interestingly, the nonspecific resource competition function (N) now gave a much better fit than the antigen-specific resource competition function (A), and was the only competition function that could not be significantly improved by extension with another competition term. Extension with a term accounting for nonspecific resource competition significantly improved the fit of the antigen-specific competition function (A  $\rightarrow$  NA). Thus, nonspecific resource competition must have played a significant role in the second experimental approach. Increasing the antigen availability by increasing the concentration of presented peptides on APCs (approach 2) apparently differs from increasing the antigen availability by increasing the concentration of APCs presenting peptides (approach 1). Our interpretation is that T



cells are competing for antigenic sites on APCs, *i.e.* for spaces on APCs where T cells can bind to their specific antigen without being disturbed by surrounding T cells. Because the concentration of APCs was fixed in the second experimental approach, there was a limited number of APC sites T cells could bind to. Prepulsing with higher peptide doses might thus have increased the peptide concentration *per antigenic site*, but might have failed to increase the actual antigen *availability* for T cells.

In the second experimental approach, it would be more appropriate to explicitly model the concentration of antigenic sites  $A$  as a saturation function of the peptide concentration used for prepulsing the APCs (see Appendix). When such a saturation is substituted in equation (4), a proliferation function is obtained that has the same qualitative behaviour as the nonspecific resource competition of equation (5), explaining why the latter function described the data better than the antigen-specific resource competition function of equation (4). Because in the first experimental approach the number of antigenic sites  $A$  increased linearly with the concentration of APCs, both functions gave a good fit to the data in Figure 1. Additionally, the fact that the data fitting in our two experimental approaches gave qualitatively different results suggests that T cells were not merely competing for resources such as growth factors or nutrients in the medium. If T cells were competing for such APC-independent resources, one would expect Figures 2 and 4 to be similar. Finally, when the analysis was repeated with arthritogenic A2b T cells, the same qualitative picture as Figures 2 and 4 was obtained. This confirms that T cells compete for antigenic sites on APCs.

## Conclusion

By mathematical analysis of data from T cell proliferation assays in which both the concentration of antigen-presenting sites and the concentration of T cells were varied, we have shown evidence for T cell competition *in vitro*. Our results are in full agreement with *in vivo* data demonstrating that the proliferative capacity of T cells is influenced not only by the antigen availability but also by the presence of other T cells, *i.e.* T cell competition [80, 215, 216]. In theoretical models of the immune system, T cell competition terms are often applied for their stabilizing effect on T cell population sizes [66]. The results presented here provide an experimental validation for the use of such T cell competition terms.

Our mathematical approach enabled us to discriminate between three qualitatively different forms of T cell competition, *i.e.* direct inhibitory T–T interactions, antigen-specific resource competition, and nonspecific resource competition. The best description of the experimental data was obtained with proliferation functions involving resource competition. Comparison of two different experimental approaches, in which the antigenic ligand concentration was controlled differently, indicated that T cells were mainly competing for antigenic sites on APCs. T cell competition for antigenic sites thus seems to be a phenomenon arising naturally from the interactions of T cells with

their ligands, which should be taken into account in both experimental and theoretical studies of T cell proliferation. The relative importance of competition for a site on an APC, and competition between cells binding the same MHC–peptide complex, remains to be elucidated by developing models and experiments involving multiple T cell clones competing for multiple antigens.

## Appendix

### Derivation of the antigen-specific competition function

The T cell interaction scheme (3) can be described by the following differential equation for the T cell–antigen complexes  $C$ :

$$\frac{dC}{dt} = k_1 T_f A_f - k_{-1} C. \quad (6)$$

Following Borghans *et al.* [32], we make a quasi-steady state (QSS) approximation for the T cells in complex ( $C$ ), and substitute the equations for the total concentration of T cells  $T = T_f + C$ , and the total concentration of antigenic sites  $A = A_f + C$  into equation (6), giving:

$$\frac{dC}{dt} = k_1 \left( (T - C)(A - C) - KC \right) = 0, \quad \text{where } K = \frac{k_{-1}}{k_1}. \quad (7)$$

If the concentration of T cells and antigens in complexes  $C$  is small [32] compared to the total concentration of T cells  $T$ , and compared to the total concentration of antigenic sites  $A$ , the  $C^2$  terms in equation (7) can be neglected, yielding:

$$C \approx \frac{AT}{A + cT + K}, \quad \text{where } c = 1. \quad (8)$$

According to Huisman & De Boer [102], this  $c$  value becomes a parameter that may deviate from  $c = 1$  if T cell proliferation is modelled as a multistep process in which a T cell–antigen complex ( $C$ ) first becomes an activated T cell, which subsequently proliferates to form two free T cells ( $T_f$ ). For maximal simplicity, we have left out this activated T cell stage, but we do allow for  $c$  being a parameter that can be estimated freely. Since total T cell proliferation is assumed to be proportional to the total number of T cell–antigen complexes  $C$ , total [ $^3\text{H}$ ]-thymidine incorporation can be modelled by  $T^* = \rho C$ , *i.e.* by equation (4).

### Transforming peptides and APCs into antigenic sites

To become stimulated, a T cell has to bind to an APC and interact with the appropriate MHC–peptide complex. Because only a limited number of T cells can bind to one APC at any time, T cells compete for “sites” on APCs where T cells can bind and antigen is presented [65]. In the first experimental approach, the antigenic site concentration ( $A$ ) is increased by adding APCs, and is thus proportional to the APC concentration:  $A = nA_c$ . Here  $A_c$  represents the concentration of APCs and  $n$  the number of antigenic sites per APC. In the second approach, however, it is more appropriate to model the antigenic site concentration as a saturation function of the peptide concentration:

$$A = m \frac{A_p}{A_p/h + 1}, \quad (9)$$

with  $A_p$  denoting the peptide concentration used to prepulse the APCs,  $h$  denoting the peptide concentration at which the antigenic site concentration is half-maximal, and  $m$  representing the number of antigenic sites established per  $\mu\text{g}$  of peptide at low peptide concentrations. Because substitution of equation (9) into the antigen-specific resource competition function of equation (4) yields a competition function that is very similar to the nonspecific resource competition function of equation (5), but involves one more parameter than equation (5), we refrained from substituting equation (9) when we fitted the experimental results of Figure 3. Instead we substituted the linear domain of equation (9), *i.e.*  $A = mA_p$ .

Since both  $n$  and  $m$  are unknown, we have scaled the  $c$  and  $K$  parameters by dividing both the numerator and the denominator of the proliferation functions of equations (1), (2), (4) and (5) by  $n$  in the first experimental approach, and by  $m$  in the second approach. In the fitting procedure we have thus estimated  $c_1 = c/n$  and  $K_1 = K/n$  for the first experimental approach and  $c_2 = c/m$  and  $K_2 = K/m$  for the second experimental approach. This explains the differences in  $c$  and  $K$  in the first and second experimental approach (see the legend of Table 1 for further details).

We thank Lex Borghans and Jorge Carneiro for useful discussions.



# 8

## Summarizing discussion

## Different sources of diversity

This thesis addresses various sources of diversity in the vertebrate immune system. In particular, we have studied the diversity employed by lymphocytes, which are responsible for the *recognition* of antigens, and the diversity of major histocompatibility (MHC) molecules, which are responsible for the *presentation* of antigens to the immune system. In principle, lymphocytes and MHC molecules are involved in the same task, *i.e.* to allow immune responses to many foreign antigens, while avoiding inappropriate responses such as autoimmunity. Given the diversity of foreign and self molecules, it is perhaps not surprising that both MHC molecules and lymphocytes have a high degree of diversity. Nevertheless, they differ fundamentally in the level at which their diversity is expressed. While any vertebrate individual expresses a huge diversity of B and T lymphocytes, the diversity of MHC molecules is mainly evident at the population level. This suggests that MHC and lymphocyte diversity play quite distinct functional roles.

## Evolutionary and somatic learning

Central to this thesis is the hypothesis that the adaptive immune system stores immunological decisions in lymphocytes. The decision as to which type of immune response to induce against an antigen is based on the context of the antigen, *e.g.* its localization [234], any tissue damage caused by the antigen [135], and/or signals from the innate immune system [104]. If effector or memory clones recognize a subset of the epitopes that are expressed by an antigen, they too form part of the antigen context, and provide information on the type of immune response that is to be induced. Being fairly independent of costimulatory signals, such instructed lymphocytes help to eliminate pathogens upon re-encounter even before any tissue damage has been done, and help to induce appropriate immune responses against new antigens that correlate with previously encountered antigens. The vertebrate immune system thus combines the evolutionary wisdom of the innate immune system with somatic learning by the adaptive immune system.

Somatic learning by the adaptive immune system has an illustrative analogy with the way the brain learns about fear. A consequence of “learned fear” is that the body reacts as if it is in danger even if a situation “doesn’t contain anything that is intrinsically threatening, yet is bristling with signs you have learned to associate with danger” [16]. We think this concept applies to lymphocytes as well. Even without an inflammatory context, effector and memory lymphocytes recognizing certain epitopes of an antigen will associate the antigen with previously encountered antigens and respond accordingly. As we have shown in Chapters 2–4, this has important implications for the diversity of lymphocytes and MHC molecules.

## Diversity of lymphocytes

There is good evidence that the adaptive immune system of vertebrates evolved when recombination-activating genes (RAGs) became inserted into a vertebrate receptor gene [4, 95], enabling recombination of parts of the genome, and thereby expression of a huge diversity of specific lymphocytes [10]. It has been suggested that the diversity of the vertebrate immune system arose as a side-effect of the genetic coding for antigen-receptors. Using an evolutionary model it has been shown that immune systems with somatic recombination naturally evolve a large repertoire diversity to respond to a wide variety of pathogens. Under similar conditions, in immune systems lacking somatic recombination the number of different receptors naturally decreases [213].

Alternatively, it has been proposed that the need for self–nonself discrimination is the driving force for the diversity of the adaptive immune system. Using mathematical models it has been shown that the diversity of lymphocytes giving optimal protection against infections reflects the number of self antigens that need to be tolerized [62, 152, 228]. We have shown in Chapter 2 that avoidance of inappropriate immune responses calls for an even higher specificity and diversity than was concluded from these previous models. Because a significant part of all self epitopes fails to induce self tolerance [50, 169, 185], cross-reactive lymphocytes would run the risk of being triggered by foreign antigens and subsequently turning autoaggressive against so-far ignored self antigens [12, 159, 160, 232]. Likewise, lymphocytes that have been instructed to mount a particular type of immune response against an antigen should not cross-react with other antigens that normally invoke a different type of immune response. According to our calculations, lymphocytes should be as specific as possible within the constraints imposed by the size of the immune repertoire. (Large) vertebrates with large immune repertoires would therefore be predicted to have more specific lymphocytes, and hence a smaller chance to suffer from infections and autoimmune diseases, than (small) vertebrates with small immune repertoires.

We have studied the storage of appropriate responses by lymphocytes in Chapter 3, and found that the advantages conferred by an adaptive immune system outweigh its disadvantages whenever (i) the immune repertoire is sufficiently specific, and (ii) there is some correlation in terms of overlapping epitopes between antigens that require similar types of immune response. If the adaptive immune system were insufficiently specific, the immune system would be better off without it, because inappropriate immune responses would tend to be induced. Lymphocyte diversity reconciles specificity (required to avoid inappropriate responses) with reactivity (required to respond to many antigens). We have shown that the contribution of memory lymphocytes in immune responses to new antigens is not hampered by increasing the size of the immune repertoire. One therefore expects evolution to select for highly diverse and specific lymphocyte repertoires.

## Diversity of MHC molecules

In contrast to lymphocytes, MHC molecules bind their ligands degenerately. Nevertheless, hundreds of different MHC molecules have been observed, albeit only at the population level [166, 223]. Individuals express only a small fraction of this MHC diversity [167]. In Chapter 4, we have disputed the widely held view that the individual diversity of MHC molecules is limited to avoid T cell repertoire depletion during self tolerance induction [54, 62, 106, 157, 164, 211, 222]. Re-examining a previously proposed model [157], we have shown that expression of extra MHC molecules tends to increase the functional T cell repertoire and that repertoire depletion only occurs at an unrealistically high individual MHC diversity. Additionally, we have demonstrated that the selection pressure for a larger MHC diversity within an individual fades away once there are of the order of ten different MHC molecules per individual. Expression of a larger individual MHC diversity has the added disadvantage that the chance of inducing inappropriate immune responses increases. Foreign peptides presented by MHC molecules may form complexes that — from the T cell point of view — look similar to complexes of MHC molecules presenting ignored self molecules. Excessive MHC diversity therefore increases the chance that lymphocytes that have been triggered by foreign peptides cause autoimmune responses against so-far ignored self antigens.

By simulating the evolution of hosts and pathogens, we have demonstrated in Chapter 5 that a large polymorphism of MHC molecules naturally arises in host populations infected by pathogens. The simulations show that there is selection favouring heterozygosity at the MHC loci [68, 99–101, 212]. If the hosts and pathogens in our simulations coevolve, there is frequency-dependent selection in addition, which favours the expression of rare MHC molecules [19, 27, 195, 202]. Rare MHC molecules tend to provide protection against pathogens that avoid presentation by the most common MHC molecules in the population. We have shown that the MHC polymorphism arising under host–pathogen coevolution is significantly larger than the polymorphism arising under selection for heterozygosity only. In the simulations of Chapter 5 there is no explicit disadvantage of expression of many different MHC molecules per individual, as we proposed in Chapter 4. If a disadvantage of a high individual MHC diversity is taken into account, the effect of heterozygosity selection on the MHC polymorphism diminishes at a sufficiently high individual diversity. Heterozygosity selection works by favouring the individuals with the largest MHC diversity. Selection for expression of rare MHC molecules, on the other hand, would remain.

For other defence systems, *e.g.* the restriction–modification (RM) system which protects bacteria against invading genetic material, two modes of diversity have been described: an individual-based mode in which every bacterium expresses all possible RM specificities, and a population-based mode in which each bacterium expresses maximally one RM system, with the total set of RM systems being expressed at the population level [162]. Pagie & Hogeweg [162] demonstrated that such a population-based mode even exists in the absence of any costs for expression of RM systems. Expressing a limited number of defence systems per individual allows individuals to be different from each



other. Analogously, the population diversity of MHC molecules allows different vertebrate individuals to respond differently to identical antigens, thereby giving protection against coevolving pathogens. The MHC diversity within any individual allows vertebrates to reconcile this population diversity with presentation of many antigens.

## Diversity of immune responses

Upon antigenic challenge, MHC molecules and T lymphocytes aggregate at the cell surface of APCs to enable the induction of an appropriate response. During the T cell–APC contact — a dynamic interaction [219] which typically lasts several hours [117] — the engaged T cell receptors (TCRs) and MHC–peptide complexes are temporarily unavailable for other T cell–APC contacts. In Chapter 6 we have derived a T cell proliferation function accounting for the temporary unavailability of both antigen and TCRs. We have applied this proliferation function in Chapter 7, and have shown evidence for competition between T lymphocytes of the same specificity for antigen-presenting sites on APCs. If different lymphocyte clones were to compete for a single ligand, the clone with the highest affinity for that ligand would be expected to outcompete all other clones [63]. T cell competition would thereby jeopardize the reliable storage of appropriate types of immune response in multiple lymphocyte clones, which is needed for recognition of correlated antigens. We conjecture that the immune system may employ its MHC diversity at the individual level to allow the presentation of multiple epitopes per antigen, and thereby to prevent immune responses from becoming monoclonal.

In summary, this thesis demonstrates that it is neither the pathogen diversity nor the self diversity, but instead the need to avoid inappropriate immune responses, that can explain the diversity in the immune system. For example, avoidance of autoreactivity to self molecules that fail to induce self tolerance can explain both the enormous individual diversity of lymphocytes and the limited individual diversity of MHC molecules. The individual MHC diversity that is left may play a role in the distributed storage of immunological decisions in the presence of T cell competition. The diversity of coevolving pathogens does explain the diversity of MHC molecules at the population level.



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## Abbreviations

APC	antigen-presenting cell
cpm	counts per minute
FCS	fetal calf serum
HSP	heat shock protein
LCMV	lymphocytic choriomeningitis virus
MBP	myelin basic protein
MHC	major histocompatibility molecule
QSSA	quasi-steady state assumption
rQSSA	reverse QSSA
tQSSA	QSSA of the total substrate model
RAG	recombination-activating gene
SSR	sum of the squared residuals
TCR	T cell receptor
Th1	T helper type 1
Th2	T helper type 2
VV	vaccinia virus



## Samenvatting

Het immuunsysteem kenmerkt zich door zijn grote diversiteit. Miljarden witte bloedcellen (lymfocyten), elk met andere receptoren op het celoppervlak, bieden bescherming tegen ziekteverwekkende parasieten, bacteriën en virussen (pathogenen). Tijdens infecties vermenigvuldigen zich alleen de lymfocyten die specifiek het infecterende pathogen herkennen, zodat het pathogen kan worden verwijderd en het lichaam beter beschermd is als het een tweede keer door hetzelfde pathogen geïnfecteerd wordt. Omdat lymfocyt-receptoren grotendeels aselekt worden samengesteld, bestaat het risico dat lymfocyten zich richten tegen lichaamseigen moleculen. Om zulke autoimmunereacties te voorkomen worden lymfocyten die lichaamseigen moleculen herkennen verwijderd of geïnactiveerd. Het immuunsysteem leert zo lichaamseigen van lichaamsvreemde stoffen te onderscheiden.

Behalve door de enorme diversiteit aan lymfocyten kenmerkt het immuunsysteem zich ook door de veelheid aan verschillende *typen* immunereacties. Zo induceert een virale infectie in de long een heel ander soort immunereactie dan een bacteriële infectie van de darm. Een in het oog springend verschil in immunereacties is het onderscheid tussen B- en T-cel-acties. B-lymfocyten herkennen pathogenen in hun natuurlijke vorm en produceren antilichamen als ze een pathogen herkennen. T-lymfocyten daarentegen, herkennen pathogenen alleen nadat stukjes eiwit (peptiden) van pathogenen gepresenteerd zijn op zogenaamde *major histocompatibility* (MHC-) moleculen op het oppervlak van antigeen-presenterende cellen.

Ook deze MHC-moleculen staan bekend om hun grote diversiteit. MHC-moleculen verschillen vooral daar waar peptides worden gebonden. Verschillende MHC-moleculen induceren daardoor verschillende T-cel-acties. In tegenstelling tot de diversiteit van lymfocyten komt de diversiteit van MHC-moleculen vooral tot uitdrukking op populatieniveau en niet zozeer op individueel niveau. In elk individu zorgen grofweg tien verschillende MHC-moleculen voor de presentatie van pathogenen aan het immuunsysteem. Op populatieniveau zijn echter honderden verschillende MHC-moleculen gevonden. Doordat praktisch elk individu een andere set MHC-moleculen tot expressie brengt, verschillen individuen in hun immunereacties tegen identieke pathogenen.

In dit proefschrift worden wiskundige modellen en computersimulaties gebruikt om te onderzoeken welke evolutionaire selectiedruk de diversiteit van lymfocyten en MHC-moleculen kan verklaren. De grote individuele diversiteit aan lymfocyten werd veelal gezien als een aanpassing aan de grote diversiteit aan pathogenen waaraan gastheren worden blootgesteld. De modellen in dit proefschrift laten echter zien dat niet de afweer tegen veel verschillende pathogenen maar juist het voorkómen van ongewenste immunereacties een hoge diversiteit van lymfocyten vereist. Een centrale hypothese in dit proef-

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schrift is dat lymfocyten die eenmaal een peptide herkend hebben, en geïnstrueerd zijn over het type immunreactie dat ze moeten induceren, dit fenotype “onthouden.” Wanneer ze hun peptide opnieuw tegenkomen “weten” dergelijke lymfocyten dus al welk type immunreactie gemaakt moet worden. Een gevaar van zo’n somatisch lerend immuunsysteem is dat geïnstrueerde lymfocyten een verkeerd soort immunreactie teweeg kunnen brengen als ze ook reageren op peptides van pathogenen die een ander soort immunreactie vereisen. De modellen in dit proefschrift laten zien dat er daardoor een evolutionaire selectiedruk is op de expressie van een zo specifiek en divers mogelijke set van lymfocyten per individu.

Gezien de rol van MHC-moleculen in immunreacties is het verbazingwekkend dat de individuele MHC-diversiteit zo klein is vergeleken met de populatiediversiteit van MHC-moleculen. Een veelgebruikte argumentatie hiervoor is dat een beperkte MHC-diversiteit per individu voorkómt dat het T-cel-repertoire uitgeput raakt tijdens zelf-tolerantie-inductie. Dit proefschrift laat echter zien dat dit verbale argument niet kan werken. Een negatief effect van MHC-moleculen op het T-cel-repertoire blijkt pas op te treden bij een onrealistisch hoge individuele MHC-diversiteit. De modellen suggereren twee alternatieve verklaringen voor de beperkte individuele MHC-diversiteit. Allereerst vervalt de selectiedruk voor een grotere individuele MHC-diversiteit zodra het aantal verschillende MHC-moleculen per individu hoog genoeg is om de presentatie van willekeurige pathogenen te kunnen garanderen. Ten tweede bieden extra MHC-moleculen een verhoogd risico op het ontstaan van ongewenste immunreacties, zoals bijvoorbeeld immunreacties tegen lichaamseigen moleculen die er niet in geslaagd zijn zelf-tolerantie te induceren.

Ondanks de selectiedruk tegen een te hoge individuele MHC-diversiteit, blijft er selectie voor de expressie van MHC-moleculen die binnen de populatie weinig voorkomen. Pathogenen kunnen dankzij hun relatief snelle evolutie “leren” om niet gepresenteerd te worden op MHC-moleculen die algemeen voorkomen in de populatie. Door anders te zijn dan de andere individuen binnen een populatie verkrijgt een gastheer bescherming tegen zulke evoluerende pathogenen.

Hoewel zowel lymfocyten als MHC-moleculen betrokken zijn bij dezelfde taak, namelijk het opbouwen van immuniteit tegen pathogenen en het voorkómen van autoimmuniteit, is er dus een fundamenteel verschil in hun diversiteit. Dit proefschrift laat zien dat zowel de grote individuele diversiteit van lymfocyten als de lage individuele diversiteit van MHC-moleculen kan worden verklaard uit de noodzaak ongewenste immunreacties zoals autoimmunreacties te voorkomen.



## Curriculum Vitae

José Borghans was born on January 1st, 1972 in Geleen, The Netherlands. In 1990 she attended “Scholengemeenschap Sint Michiel” in Geleen where she gained an Atheneum B diploma in 1990. In that same year she began reading Biology at Utrecht University, where she obtained a Master’s degree (doctoraal, cum laude) specialising in Theoretical Biology and Immunology. She was awarded a 1996 Unilever Research Prize for her final year research work. In 1994 she also obtained the first-year examination (propedeuse, cum laude) in Mathematics at Utrecht University. From April 1996 until April 2000 she worked as a postgraduate student in the Theoretical Biology group at Utrecht University, where she carried out the research described in this thesis. From July 2000 she will work as a postdoctoral researcher in the “Laboratoire des Dynamiques Lymphocytaires” at the “Institut Pasteur” in Paris.



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