Analysis of the CD4+ T cell immune response in vivo using CD4 up-regulation as an activation marker
normal versus autoimmune peripheral CD4+ T cell response development

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Publication Date: 1999
Permanent Link: https://doi.org/10.3929/ethz-a-003840267

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ANALYSIS OF THE CD4⁺ T CELL IMMUNE RESPONSE IN VIVO USING CD4 UP-REGULATION AS AN ACTIVATION MARKER:
NORMAL VERSUS AUTOIMMUNE PERIPHERAL CD4⁺ T CELL RESPONSE DEVELOPMENT

A Dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY, ZURICH

For the degree of
Doctor of Natural Sciences

presented by
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1999

The experimental part of this thesis was performed in the laboratory of Prof. Dr. C. Garrison Fathman under the supervision of Prof. Dr. William M. Ridgway, Department of Medicine, Division of Immunology and Rheumatology, Stanford, California, USA.
Ai Miei Cari Genitori
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SUMMARY

The primary aim of adaptive immunity is to combat and eradicate "pathogens" without compromising host integrity. During the processes of adaptive immunity, naive antigen-specific CD4+ T lymphocytes become activated through specific interaction with their ligand, antigenic peptide bound to a major histocompatibility (MHC) class II molecule, and interaction with requisite "co-stimulatory" molecules. The activated CD4+ T cells then proceed to expand and develop into T helper (Th) cells with unique effector functions. After the pathogen disappears, multiple regulatory mechanisms function to return the immune system to its basal state, while immunological "memory" protects the host from re-encounter with the same antigen. Due to the difficulty of tracking the small number of antigen-specific T cells in vivo, our present knowledge of in vivo antigen-specific T cell development has been confined to the study of Th-cell responses of T cells with fixed T cell receptors (TCR-transgenic T cells) or to peptide-specific responses dominated by T cells using restricted sets of antigen specific receptors.

In this thesis a system was developed to investigate the T cell response of antigen-specific T cells in vivo, which overcame the problem of low precursor-frequency. This system is based on culturing under limiting dilution conditions antigen-specific T cells directly after their isolation from lymph nodes of primed animals, using CD4^high as a newly identified marker for activated T cells in vivo. Panels of T cell clones, obtained by limiting dilution cloning of the CD4^high T cells, allowed the study of changes which occurred during the course of the CD4^+ T cell immune response in vivo.

Our initial studies demonstrated that antigen-specific murine CD4+ T cells up-regulated their surface CD4 expression following antigen recognition. Characteristics of CD4 up-regulation were studied in response to the antigen Sperm Whale Myoglobin (SWM) amino acids 110-121 in DBA/2 mice. Flow cytometry and limiting dilution analysis (LDA) showed that the CD4^high cells contained all of the antigen-specific T cells both in vitro and in vivo.

Having shown the usefulness of CD4^high as a marker for activated T cells, limiting dilution analysis of CD4^high T cells was used to follow an immune response in vivo. CD4^high T cells from primed lymph nodes of DBA/2 mice were isolated at various time points after primary and secondary immunization with SWM 110-121, and directly cultured under limiting dilution conditions. This strategy resulted in enumeration of the number of CD4^+ T cells responding to antigen over time and provided a broad panel of "LDA-T cell clones" characteristic for each time point after immunization. CD4^+ T cell receptor sequence and proliferation analyses of "LDA-T cell clones" suggested that a diverse antigen-specific CD4^+ T cell repertoire was present 3 days after immunization, but rapidly, within 6 days following immunization, a population of intermediate
Summary

affinity T cells emerged which persisted for at least four weeks following primary immunization and dominated the secondary response. These data provided evidence for a T cell affinity selection mechanism acting early in the immune response in vivo and suggested that expansion and persistence of a precursor pool of intermediate affinity T cells defined CD4+ T cell memory.

This technique also allowed us to study the development of Th1 and Th2 T cell subsets during an immune response in vivo. The cloning strategy mentioned above yielded a set of T cells with a broad range of affinities for a specific antigen, and offered a method to test the in vivo relevance of an avidity model for CD4+ T cell differentiation. Rather than varying the affinity of peptide for the TCR or the MHC, our system allowed to maintain the structure and density of the [peptide:MHC] complex constant, only varying the affinity of the TCR in a population of LDA-T cell clones. Intracellular cytokine staining revealed a Th2 phenotype in low affinity T cells, while intermediate/high affinity T cells displayed a Th1-like phenotype, demonstrating a direct correlation between TCR affinity for the priming antigen and the development of a Th1 or Th2 response in vivo.

We next asked whether the affinity of the pre-existing antigen-specific T cell repertoire influenced Th1/Th2 differentiation of the immune response. This question was addressed by analyzing the (B6xA)F1 response to self and two foreign species variants of mouse myoglobin determinant 67-79, which were shown to bind with similar affinities to the restricting MHC class II molecules. While the T cell repertoire reactive to the foreign antigens showed a high affinity for the [peptide:MHC] complex and lead to a Th1 response in vivo, the self-reactive T cell repertoire had a low affinity and resulted in the development of Th2 responses.

The examination of immune responses against foreign and self antigens was extended to autoimmune models of disease. To guarantee survival of the host, it is essential that antigen-specific T cell responses only occur in response to foreign antigens. A number of self-tolerance-inducing mechanisms exist to protect the host from autoimmune attack. The MHC is one major genetic factor predisposing to (or protecting from) autoimmunity. One current paradigm for the association of MHC with autoimmune diseases suggests that efficient binding of autoantigens by disease-associated MHC molecules leads to a T cell-mediated autoimmune response. On the basis of results obtained in an animal model of autoimmune diabetes, the NOD mouse, a new model for MHC and disease association was proposed, where susceptibility-linked MHC molecules lead to altered thymic selection resulting in the escape of high-affinity self-reactive (potentially autoreactive) T cells into the periphery.

In conclusion, this work offers a novel understanding of the development of self versus foreign specific CD4+ T immune responses in both normal and autoimmune disease models.
ZUSAMMENFASSUNG


Diese Arbeit führt zu einem verbessertem Verständnis der Entwicklung von selbst- vs. fremdspezifischen T-Helfer-Immunantworten sowohl in naiven Tieren als auch in Mausmodellen für Autoimmunerkrankungen beizutragen.
GENERAL INTRODUCTION

The development of both an innate and an adaptive immune system has provided higher vertebrates with an exceptional defense mechanism capable of responding to a variety of foreign invaders without responding to self-antigens.

The characteristic trait of the innate immune system is its immediate availability upon exposure to a pathogen. It is composed of natural physical barriers like the skin and mucous membranes and is mediated by macrophages, granulocytes, natural killer cells and by soluble factors like cytokines, chemokines, and complement. This natural defense mechanism, however, cannot discriminate between foreign and self-antigens and does not confer life-long protective immunity upon re-exposure to the same antigen.

Specificity, diversity and memory are three essential and critical features of our immune system which are provided by a second defense mechanism, the adaptive immunity.

First, specificity is conferred by a large number of T and B Lymphocytes, each bearing an unique antigen receptor of exquisite specificity for a particular antigen (Burnet, 1959; Evavold et al., 1995; Kersh and Allen, 1996; Sloan-Lancaster and Allen, 1996). Second, in order for these antigen-specific receptors to match the enormous variety of antigens present in the hostile environment, antigen receptor diversity is generated by a random mechanism (Davis, 1990; Davis and Bjorkman, 1988; Tonegawa, 1983) allowing the immune system to recognize and eliminate any possible foreign intruder without damaging the host itself. Finally, the adaptive immune system is capable of “remembering” any previous antigenic challenge, a phenomenon called “immunologic memory” (Ahmed and Gray, 1996; Gray, 1993; Gray, 1994; Gray et al., 1996; Kündig et al., 1996; Mackay, 1993; Sprent and Tough, 1994; Vitetta et al., 1991; Zinkernagel et al., 1996; Zinkernagel et al., 1997), such that, upon re-exposure with the same antigen, a faster and stronger immune response occurs.

In healthy individuals, the immune system does not react destructively against self, a phenomenon called self-tolerance. When under particular circumstances self-tolerance is abolished, the immune system reacts destructively against the host resulting in autoimmune sequelae. Self-tolerance may involve any one of the following mechanisms: central deletion (Bluthmann et al., 1988; Burnet, 1959; Kisielow et al., 1988; Kurts et al., 1996; Kurts et al., 1997; Lederberg, 1959), central unresponsiveness (Blackman et al., 1990; Kawai and Ohashi, 1995; Rammensee et al., 1989; Ramsdell et al., 1989; Roberts et al., 1990; Schonrich et al., 1992), peripheral clonal deletion (exhaustion) (Arnold et al., 1993; Rocha et al., 1993; Webb et al., 1990; Zinkernagel et al., 1997), clonal ignorance (Aichele et al., 1996; Hammerling et al., 1991; Lo et al., 1991;
Zinkernagel, 1996; Zinkernagel et al., 1993), peripheral unresponsiveness (Perez et al., 1997; Ramsdell et al., 1989; Roberts et al., 1990), immunoregulation (Powrie et al., 1994; Sakaguchi et al., 1985; Saoudi et al., 1996) and/or immune deviation (Rocken and Shevach, 1996). Conversely, the induction of autoimmunity may include mechanisms like molecular mimicry (Fujinami and Oldstone, 1985; Ohashi et al., 1993; Wucherpfennig and Strominger, 1995), induction of cytokines (Liblau et al., 1995; O'Garra and Murphy, 1993; O'Garra et al., 1997; Powrie and Coffman, 1993), induction of inappropriate costimulation (Campbell et al., 1989; Campbell et al., 1988; Green et al., 1998; Neufeld et al., 1989; Parkkonen et al., 1992) or induction of antigen processing and presentation (Kaufman et al., 1993; Lehmann et al., 1992; Lehmann et al., 1993; Opdenakker and Van Damme, 1994; Tisch et al., 1993). Population, family and twin studies have established that genetic factors greatly influence predisposition for autoimmune disease, with the most significant genetic association being related to the major histocompatibility complex (MHC) (Nepom, 1993; Nepom and Erlich, 1991; Nepom et al., 1987; Scharf et al., 1989; Sollid et al., 1989; Tiwari and Terasaki, 1985).

The studies presented in the following pages are intended to elucidate some of the mechanisms by which normal versus autoimmune CD4+ T cell-mediated immune responses develop.

Therefore, the rest of this introduction will principally center on those aspects of the adaptive immunity which are required to fully understand the significance of the data presented. The major focus will be directed towards a particular cell subset involved in cellular immunity, the CD4+ T helper lymphocytes, and the events which both precede and follow their encounter with a self or foreign antigen.

1. Generation of T lymphocyte diversity

One branch of the adaptive immune system includes T lymphocytes, which are the mediators of cellular immunity. Most T lymphocytes (90%-95%) express antigen receptors consisting of disulfide-linked heterodimers of αβ polypeptide chains (Dembic et al., 1986; Hedrick et al., 1984). The remaining small subset of T lymphocytes that express γδ chains (Chien et al., 1987; Chien et al., 1996; Hayday et al., 1985) will not be discussed here. The TCR αβ heterodimer confers upon T cells the ability to recognize their ligand, peptide antigens bound to MHC molecules, while the actual signal transduction occurs through proteins of the CD3 complex,
the γ, δ, ε and ζ chains, which are non-covalently bound to the intracellular region of the TCR αβ chains (Terhorst et al., 1996).

Like Ig genes in B cells, the TCR α and β chain genes are composed of distinct segments that are joined by somatic recombination during T cell development (Tonogawa, 1983). For the α chain, one out of about 100 variable (Va) gene segments rearranges to one out of about 50 joining (Ja) gene segments to create a complete V-domain exon. For the β chain, a functional V-domain exon is formed by the rearrangement of one out of 30 Vβ gene segments with one out of 2 diversity (Dβ) and one out of 12 Jβ gene segments. The VJa exon is then transcribed and spliced to join the constant gene segment Ca (Raulet et al., 1985; Snodgrass et al., 1985) while the VDJβ exons are transcribed and spliced to either the Cβ 1 or the Cβ 2 exons (Raulet et al., 1985; Toyonaga and Mak, 1987). Finally, the resulting mRNAs are translated to form the α and β chain proteins. Unlike Immunoglobulins, TCR heterodimers are not secreted. Allelic exclusion, a phenomenon describing the inhibition of rearrangement of an allelic locus on one chromosome by the productive rearrangement of the corresponding allelic locus on the other chromosome, only occurs in the TCRβ chain locus (Uematsu et al., 1988). Therefore, it is possible to find T cells expressing two types of TCRα chains on their surface (Borgulya et al., 1991; Casanova et al., 1991; Hardardottir et al., 1995; Heath and Miller, 1993).

The combination of different molecular mechanisms is responsible for the generation of high structural diversity of TCR heterodimers (Davis and Bjorkman, 1988). These mechanisms are similar to the mechanisms that generate antibody diversity and consist of the use of multiple germline V, D, J segments and their combinatorial association to create TCR of different specificities. Moreover, additional diversity at VJ, VD and DJ junctions is generated through the random addition of nucleotides that are not part of the genomic sequence (N-region diversification), which is catalyzed by the enzyme terminal deoxyribonucleotidyl transferase (TdT) (Quertermous et al., 1986; Siu et al., 1984). Due to the uncommon feature of Dβ segments, which often can be translated in all three reading frames (Kronenberg et al., 1986), this imprecise joining still often leads to functional rearrangements. The combination of these different mechanisms of diversity generation could lead to a potential T cell repertoire ranging from $10^{10}$ to $10^{16}$ different specificities. In comparison, the size of the potential B cell repertoire has been estimated to reach $10^{13}$ different specificities. This size difference is mainly due to the greater number of J segments and the greater junctional diversity in the TCR genes (Berek and Milstein, 1988; Davis and Bjorkman, 1988).
2. Mechanistic models for TCR-ligand specificity and recognition

Although structurally TCRs are very similar to Igs (Bentley et al., 1995; Fields et al., 1995) there is an important distinction in the way B cells and T cells use their receptors to recognize antigen (Fig. 1). While Igs specific for protein antigens recognize conformational determinants that exist when proteins are in their native tertiary configuration, T lymphocytes, through their T cell receptor (TCR), recognize only linear determinants of peptides within the peptide binding cleft of histocompatibility complex (MHC) class I or class II molecules on antigen presenting cells (APC) (Bjorkman et al., 1987; Davis and Bjorkman, 1988; Maryanski et al., 1986; Townsend et al., 1985), a phenomenon known as antigen-specific MHC-restricted T cell recognition (Zinkernagel and Doherty, 1974). Peptides derived from pathogens that expand within the cytoplasm of the cells are carried to the cell surface by MHC class I molecules and presented to CD8+ T lymphocytes, which differentiate into cytotoxic T cells (CTL) and kill infected target cells. In contrast, peptides derived from endocytosed extracellular pathogens are carried to the cell surface by MHC class II molecules and presented to CD4+ T helper lymphocytes (Th), which differentiate into effector T cells releasing characteristic sets of cytokines.

The crystal structures of peptide-MHC complexes have shown that only three to four side chains of the peptide are accessible by the TCR (Bjorkman et al., 1987; Fremont et al., 1992; Madden et al., 1991; Stern et al., 1994). In spite of the limited number of side chains being recognized, TCR recognition is exquisitely specific. Studies using altered peptide ligands (APLs) have demonstrated, however, that the recognition by the TCR is rather flexible in that the same TCR can selectively respond to more than one type of peptide. APLs are analogs of immunogenic peptides, in which one or two TCR contact sites have been manipulated. They do not induce a proliferative response but can mediate other T cell functions (i.e. cytokine secretion or cytotoxicity) (Evavold and Allen, 1991; Evavold et al., 1993; Jameson et al., 1993; Sloan-Lancaster and Allen, 1996). Because APLs induce partial activation, they are also called partial agonists. Certain partial agonists (antagonists) have the capacity of inhibiting the response to an agonist peptide in dose-dependent fashion when presented simultaneously to the responding T cell on an APC (De Magistris et al., 1992; Racioppi et al., 1993), while others have been shown to induce a state of unresponsiveness (Sloan-Lancaster et al., 1993; Sloan-Lancaster et al., 1994).

The analysis of intracellular signaling events occurring in response to APLs demonstrated a distinct pattern of CD3 ζ chain phosphorylation and the failure to activate ZAP-70 kinase (Madrenas et al., 1995; Sloan-Lancaster et al., 1994). Recently, it was found that upon TCR engagement, the ζ chain undergoes a series of successive phosphorylation steps, whose completion depends on the nature of the ligand (Kersh et al., 1998).
The recent determination, at high resolution, of the structure of TCR-MHC-peptide complexes (Garboczi et al., 1996; Garcia et al., 1996) as well as recently published affinity and kinetic results from surface plasmon resonance (BIAcore\textsuperscript{TM}) measurements of the binding of the TCR to purified MHC-peptide complexes have set a quantitative basis for mechanistic models which try to explain how a ligand differing only slightly from an agonist can deliver an unique signal to the cell (Margulies, 1997; Padlan and Marguilies, 1997). Two general classes of models have been proposed, which are not necessary exclusive: allosteric and crosslinking models. Allosteric models are based on structural changes induced by the TCR by its interaction with the MHC-peptide complex, while cross-linking models are based on the spatial topological rearrangement of the TCR upon interaction with its ligand. While the available crystallographic data still have not excluded an allosteric component in TCR signaling, they do support, together with the kinetic data, a cross-linking model for T cell activation. An important results of the binding studies was to show that the affinities of the TCR for the peptide:MHC complexes are much lower than that of immunoglobulins for protein antigens, ranging from $5 \times 10^{-4}$ M to $10^{-7}$ M (Alam et al., 1996; Boniface and Davis, 1995; Corr et al., 1994; Matsui et al., 1991; Matsui et al., 1994; Sykulev et al., 1994; Weber et al., 1992). More recently, the determination of kinetic rate constants by surface plasmon resonance (BIAcore\textsuperscript{TM}) of TCR interactions with MHC complexed with altered peptide ligands revealed a striking correlation of the TCR affinity and particularly the dissociation rates for the peptide:MHC complex with the biological outcome of the T cell response (Alam et al., 1996; Davis et al., 1998; Davis et al., 1997; Lyons et al., 1996; Matsui et al., 1994; Rabinowitz et al., 1996). For example, a TCR was shown to bind to antagonist ligands with lower affinities and faster dissociation rates than to agonists (Lyons et al., 1996) while increasing off-rates of agonist ligands were shown to correlate with decreasing agonist activity (Matsui et al., 1994). The results obtained from these kinetics measurements and from the multistep TCR $\zeta$ phosphorylation data support a kinetic proof reading model for T cell activation (Grossman and Singer, 1996; McKeithan, 1995; Rabinowitz et al., 1996). This model can explain how modest differences in affinity between two different MHC:peptide complexes for the same TCR can result in distinctive responses. The length of TCR occupancy, itself determined by both off-rate and affinity for the peptide:MHC complex, could determine whether completion of $\zeta$ chain phosphorylation and any other sequence of signaling events necessary for full activation of the cell will occur (in case long-lived occupancy) or whether an arrest in multistep TCR $\zeta$ phosphorylation (in case of short-lived occupancy) will result to incomplete T cell activation (Kersh et al., 1998) (Fig. 2).

Another kinetic explanation for the phenomenon of TCR antagonism and partial agonism is based on the serial triggering model of T cell activation proposed by Lanzavecchia and colleagues.
(Valitutti et al., 1995). The observation that a few [peptide:MHC] complexes could serially engage and trigger many TCRs, suggested that ligands with lower than optimal stability could cause the too rapid triggering of functional TCRs, which could deplete the area of contact of transduction components without transducing any detectable signal (antagonists) or transduce partial signals at very high rate (partial agonists) (Valitutti and Lanzavecchia, 1997; Valitutti et al., 1995) (Fig. 3 and 4). This model also explains how T cells with such low-affinity TCRs can recognize a low number of [peptide:MHC] complexes with high sensitivity and specificity (Karjalainen, 1994).

Since the TCR and its cognate ligand interact as integral proteins on two separate cells, the formation of the TCR-[peptide:MHC] complex will be influenced by the adhesion between T cells and APCs, and the stability of the complex may be modulated by accessory molecules and shearing stress. Among the accessory molecules are the glycoproteins CD4 (Cammarota et al., 1992; König et al., 1992) and CD8 (Potter et al., 1989; Salter et al., 1990) which are expressed on mutually exclusive subsets of αβ T cells. CD4 and CD8 both serve as adhesion molecules by binding to nonpolymorphic domains of MHC class II and MHC class I, respectively, and may act as co-receptors by facilitating the TCR complex-mediated signal transduction (Rudd et al., 1988; Veillette et al., 1989). In order to attain maximal T cell activation, a second signal is required in addition to the signal provided through TCR engagement. The second signal is antigen-independent and is delivered by the interaction of CD28 on the surface of the T cell with the B7-1 or B7-2 molecules on the APC (Harding et al., 1992; Lenschow et al., 1996; Linsley et al., 1991; Linsley and Ledbetter, 1993). TCR engagement with the [peptide:MHC] ligand in the absence of the second signal will induce a state of functional unresponsiveness in the T cell, termed “anergy” (Schwarz, 1990). T cells have a second receptor for B7, called CTLA-4, which functions to terminate T cell responses (Thompson and Allison, 1997). CTLA-4 is induced on T cells after activation, and upon binding B7 it transduces signals that will inhibit IL-2 transcription and the progression through the cell cycle (Calvo et al., 1997; Krummel and Allison, 1996).

Finally, a number of adhesion molecules will facilitate TCR engagement to occur by increasing the overall avidity of cell-cell contact. The initial binding of an effector cell to its target is mediated by the antigen unspecific interaction of LFA-1 to ICAM-1/2 and CD2 to LFA-3 (Croft and Dubey, 1997; Singer, 1992).

3. Effector Th cell differentiation

Once activated, CD4+ T helper cells can collaborate with B lymphocytes for the production of antibodies and during a memory response will help inducing isotype switching of the
immunoglobulin genes (DeFranco, 1987; Parker, 1993; Rizzo et al., 1992). Activated CD4+ T helper cells can also activate macrophages to produce reactive oxygen or nitrogen intermediates as well as tumor necrosis factor alpha (TNF-α) to destroy microbes such as mycobacteria that may survive inside macrophages.

Most immune responses involve both branches of the adaptive immunity (humoral and cellular), but, in some conditions, the two types of effector reactions may also be mutually exclusive (Parish, 1972). The cytokines produced by two subsets of CD4+ T cells, Th1 and Th2 are decisive for a prevalent cellular or antibody response, respectively (Mosmann and Coffman, 1989).

Murine Th1 cells secrete interferon (IFN)-γ, interleukin (IL)-2 and lymphotoxin, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Commonly, the terms Th1 and Th2 are used to refer to responses dominated by IFN-γ (and not IL-4) or IL-4 (and not IFN-γ), respectively (Mosmann and Sad, 1996). The function of Th1 and Th2 correlate with their distinctive cytokine production: Th1 cells activate the microbicidal properties of macrophages and induce B cells to make IgG antibodies that are very effective at opsonizing extracellular pathogens for uptake by phagocytic cells (Mosmann and Coffman, 1989; Mosmann and Sad, 1996; O'Garra, 1998; Swain et al., 1991) Th2 cells initiate the humoral immune response by activating naive antigen-specific B cells to produce IgM antibodies, and may subsequently stimulate the production of different isotypes, including IgA and IgE, as well as neutralizing and/or weakly opsonizing subtypes of IgG. Th2 cells also promote mast cell and eosinophil growth, differentiation and activation (Mosmann and Coffman, 1989; Mosmann and Sad, 1996; O'Garra, 1998; Swain et al., 1991). Importantly, the production of cytokines by each T cell subset cross-regulate each other's function as well as development: IFN-γ produced by Th1 cells inhibits the development of Th2 cells as well as humoral responses (Fitch et al., 1993), whereas the production of IL-4 and IL-10 by Th2 cells inhibits Th1 development and activation as well as macrophage activation and bactericidal activity (Moore et al., 1993; O'Garra and Murphy, 1994; Paul and Seder, 1994; Sher and Coffman, 1992). Therefore, it is possible to refer to Th1 cells as cells responsible for phagocyte-dependent and Th2 cells as cells responsible for phagocyte-independent host responses (Romagnani, 1995).

It has become clear that Th1 and Th2 subsets develop from the same mature, naive CD4+ T cell precursor (Hsieh et al., 1992; Kamogawa et al., 1993; Rocken et al., 1992; Seder and Paul, 1994). Most of the factors, which have been demonstrated to control differentiation of an uncommitted T cell precursor into a Th1 or Th2 phenotype after activation, have been of environmental nature. Best characterized is the role played by cytokines at the initiation of the
immune response (Abbas et al., 1996; O'Garra, 1998; Seder and Paul, 1994) where the presence of IL-12 promotes Th1 (Hsieh et al., 1993; Seder et al., 1993) and IL-4 leads to Th2 development (Seder et al., 1992; Seder et al., 1992; Swain et al., 1991). Among the few known genetic factors (Gorham et al., 1996; Guler et al., 1996; Murray et al., 1992; Romani et al., 1993), the MHC genotype is acquiring increasing interest (Hsieh et al., 1995; Murray, 1998; Murray et al., 1992), although nothing is known about the mechanisms it could control Th1 and Th2 differentiation. Recently, different laboratories demonstrated with the use of altered peptide ligands (APL), that peptide structure and density might directly affect lineage commitment of CD4+ T cells both in vitro and in vivo (Chaturvedi et al., 1996; Constant et al., 1995; Constant and Bottomly, 1997; Hosken et al., 1995; Kumar et al., 1995; Murray et al., 1994; O'Garra, 1998; Pfeiffer et al., 1995, Schountz, 1996). Studies using peptides from myelin basic protein (MBP) (Kumar et al., 1995) or human collagen IV protein (HuCIV) (Murray et al., 1992; Pfeiffer et al., 1995) showed that increasing the affinity of the wild-type peptide for MHC class II above baseline affinity, either by mutating the peptide or by changing the genotype of the MHC, lead to enhanced priming of Th1 cells. Studies using peptides from HuCIV (Murray et al., 1992; Pfeiffer et al., 1995) or moth cytochrome c (MCC) (Tao, 1997) proteins showed that decreasing the affinity of the wild type peptide for MHC class II or the TCR below baseline affinity, lead to enhanced priming for Th2 cells (Constant and Bottomly, 1997).

4. Memory development

Although the studies presented here exclusively investigate the development of immunologic memory in the T cell compartment, the following reference to well characterized events in the development of B cell memory might be helpful to better understand some of the implications of the findings presented in this work.

4.1. B cell memory development

Humoral immunity is mediated by antibodies, which are produced by B lymphocytes. Antibodies are produced in a membrane-bound form by B lymphocytes and can specifically bind antigen (DeFranco, 1987). The primary function of antibodies is to neutralize and eliminate the antigen that induced their formation. Since antibodies are able to bind native macromolecules in soluble form, they are first directed against extracellular foreign invaders. Secretion of antibodies occurs only after antigenic stimulation (Foy et al., 1996). Different classes of antibodies, called
isotypes (IgM, IgD, IgG, IgA and IgE), are required to eliminate different types of antigens in different anatomic sites (Cohen and Milstein, 1967; Janeway et al., 1967; Tomasi and Bienenstock, 1968; Wall and Kuehl, 1983). The production of all these varied classes of antibodies is initiated by the interaction of the antigen in peripheral tissues with B cells expressing IgM and IgD on their surface, which can specifically bind the antigen (MacLennan and Gray, 1986). Following this encounter B cells undergo proliferation and differentiation into antibody-secreting plasma cells and/or long-lived memory cells (Bradley et al., 1993; DeFranco, 1987; Parker, 1993). Upon re-exposure to the same antigen, pre-expanded memory B cells are stimulated and a secondary B cells response develops which is both quantitatively and qualitatively different than the primary response (Betz et al., 1993; Rajewsky, 1996). First, the memory response develops more rapidly than the primary response and a greater frequency of antigen-specific B cells and antibodies are observed (Gray, 1993; Vitetta et al., 1991). Second, while IgM is the dominant secreted antibody isotype during the primary response, new antibodies isotypes, such as IgG, IgA and IgE, each of which is highly effective at mediating particular effector functions, are produced as a result of isotype switching of the immunoglobulin (Ig) genes (Lorenz et al., 1995; Stavnezer, 1996). Third, as a result of somatic hypermutations in their immunoglobulin genes which takes place in the germinal centers of secondary lymphoid organs (Berek and Milstein, 1988; MacLennan, 1994), an antigen-driven selection of B cells which exhibit higher affinity antibodies occurs, giving rise to affinity maturation of the immune response (Berek et al., 1991; Fish et al., 1989).

Therefore, from our current understanding, B cell-immunologic memory is mainly based on increased frequency, isotype switching and affinity selection of antigen-specific antibodies.

4.2. T cell memory development

Interestingly, while B cell memory development seems pretty well characterized, the mechanism behind T cell memory development is far from understood. The major difficulty in the study of T cell memory is how to identify and characterize antigen-specific T cells during the course of an immune response in vivo. Tracking antigen-specific T cells in vivo is a major technical problem because of the very low precursor frequency of antigen-specific T cell precursors [1/50,000-300,000 in an unprimed, 1/1000-5000 in primed mice (Ford and Burger, 1983; Gebel et al., 1983; Kojima et al., 1988; Weyand et al., 1986). The use of TCR transgenic animal circumvented this issue by providing investigators with an extensive source of antigen-specific T cells that could be used either for studies in vitro (Croft et al., 1994; Sagerstrom et al., 1993; Seder et al., 1992) or following adoptive transfers into normal recipients in vivo (Garside et al.,...
A great deal of information was obtained using TCR transgenic mouse models regarding antigen-specific clonal expansions and contractions, co-stimulation, cell cycle status, Th-differentiation and memory T cell development and maintenance. Data from Swain’s laboratory suggest that resting memory cells look very much like naive T cells in terms of limited cytokine production (producing mainly IL-2 and IL-3) while primary and memory effector cells produce the same broader range of cytokines (Bradley et al., 1993; Bradley et al., 1993). However, the question of whether the kinetics of cytokine production and cell cycle progression might be accelerated in memory CD4 or CD8 cells compared to naive T cells still remains unanswered (Dutton et al., 1998). The requirements for the generation and maintenance of memory also are still controversial. Since most of the investigations on T cell memory generation and maintenance have involved the use of TCR-transgenic cells, which express a fixed TCR, investigators were prevented from studying repertoire development or clonal maturation that may take place in vivo. Unlike B cells, T cells do not proliferate in defined anatomical sites, like the germinal centers, so investigator can not monitor changes occurring in T cells after antigen stimulation in situ, as readily as can be done for B cells. In fact, most evidence says that T cells do not require the germinal center microenvironment to establish memory (Lane et al., 1994) and do not isotype switch nor somatically mutate (Davis and Bjorkman, 1988; Hackett et al., 1992). The recent finding of a low incidence of locus-specific mutation in the TCR Vα chain of germinal center T cells by Zheng et al., has not yet been shown to play a role in the maturation of a T cell immune response (Zheng et al., 1996; Zheng et al., 1994).

A number of surface molecules have been proposed as markers for memory T cells (Gray, 1993; Mackay, 1993), although individually, none of them will exclusively distinguish memory from recently activated and effector T cell (Dutton et al., 1998). Usually, a combination of multiple criteria, including phenotype, activation state and analysis of function (i.e. cytokine production or migration patterns), have to be considered before accounting for memory (Bradley et al., 1993; Bradley et al., 1993; Dutton et al., 1998). McHeyzer-Williams et al. took advantage of a system known to display a limited TCR repertoire in response to an antigen. They used multiparameter flow cytometry to visualize antigen-responsive T cells in the minor population expressing both the dominant TCR and specific markers of T cell memory. When they noticed that the memory response to PCC was more homogeneous than the primary response in terms of CDR3 regions, they proposed that a progressive clonal maturation was taking place during the immune response. (McHeyzer-Williams et al., 1996; McHeyzer-Williams et al., 1996; McHeyzer-Williams and Davis, 1995).

Therefore, principally because of technical limitations in attempts to track very low numbers of antigen-specific T cells in vivo, the immunological question of whether T cells could
undergo a process of affinity selection during the course of the immune response in vivo has been theoretically proposed but never directly shown (De Boer and Perelson, 1994; De Boer and Perelson, 1995; Fishman and Perelson, 1995; Kelsoe, 1996; McHeyzer-Williams et al., 1996; Steele et al., 1993; Tarlinton, 1997).

5. T cell selection

T lymphocytes, like B lymphocytes, originate from bone marrow stem cells. B cells remain in the bone marrow for further differentiation, while T cells selectively migrate into the thymus where receptor gene rearrangement and T cell maturation occur. During thymic education, immature thymocytes which recognize self-MHC are selected to further mature (thymic positive selection) and exit the thymus, while T cells which react too strongly with self-peptides in the context of self-MHC are deleted (thymic negative selection). A third group of cells, which either failed to produce a productive TCR rearrangement, or whose TCR did not recognize self-MHC will die by neglect. Recent evidence indicates that after exiting the thymus the mature selected T cell pool must continuously engage self-MHC ligands in order to survive long term in the periphery (peripheral positive selection).

5.1. Positive selection in the thymus

Studies of the pattern of MHC-restriction in thymus-grafted and bone marrow chimeric mice lead to the first demonstration of positive selection by showing that that the MHC-restriction pattern of the mature T cells was determined by the particular MHC molecules expressed on thymic epithelium of the host animal in which the T cells developed (Bevan, 1977; Fink and Bevan, 1978; Sprent and Webb, 1987; Zinkernagel et al., 1978).

The use of TCR transgenic mice allowed further investigation of the process of positive selection and showed that for mature T cells to develop, immature T cell precursors have to interact via their TCR with polymorphic MHC determinants in the thymus (Berg et al., 1989; Kisielow et al., 1988; Scott et al., 1989; Sha et al., 1988; von Boehmer, 1990). Furthermore, the CD8+CD4- or CD8-CD4+ phenotype of the mature transgenic T cells after positive selection directly correlated with the TCR specificity of the original T cell clone for MHC class I or class II, respectively (Kaye et al., 1989; Teh et al., 1988). The critical role of CD4 and CD8 co-receptors in positive selection became apparent when mice lacking MHC class I genes failed to develop CD8+ T cells (Koller et al., 1990; Van Kaer et al., 1992; Zijlstra et al., 1990), while mice with disrupted MHC class II
genes could not develop CD4+ T cells (Gosgrove et al., 1991; Grusby et al., 1991). Studies with mice transgenic for the selective expression of MHC molecules in the cortical versus the medullary thymic epithelium further demonstrated the role of cortical epithelial cells in controlling positive selection (Cosgrove et al., 1992; Laufer et al., 1996; von Boehmer, 1990).

The majority of developing thymocytes (85%) are found in the thymic cortex express low to intermediate levels of CD3/TCRαβ and are "double-positive" (DP) for CD4 and CD8 expression. These cells derive from double-negative precursor (DN), which are also negative for CD3 and TCRαβ expression (1-2% of total thymocytes). Finally, 10-15% of the remaining cells are TCRαβ high /single-positive (SP) for CD4 or CD8 expression and are mostly found in the thymic medulla (Fowlkes and Pardoll, 1989; Mathieson and Fowlkes, 1984; von Boehmer, 1988). The life span of DP cells has been shown to be only 3-3.5 days (Egerton et al., 1990; Huesmann et al., 1991), during which DP require continuous TCR interaction (Brandie et al., 1994) before they can further mature to SP cells as a result of positive selection (Borgulya et al., 1991; Ohashi et al., 1990; Petrie et al., 1993; Swat et al., 1992) and migrate out of the cortex into the medulla (Crisa et al., 1996; Savino et al., 1993). If no productive interaction between the TCR and MHC determinants occurs, the DP cells will die by neglect via programmed cell death (Cohen et al., 1992; Huesmann et al., 1991; Robey and Fowlkes, 1994; Surh and Sprent, 1994; von Boehmer, 1988).

In spite of the fact that MHC alleles and TCR loci are not genetically linked, from the vast TCR diversity generated by random rearrangement mechanisms, about 20% of randomly assembled TCRs can recognize the small set of self-MHC molecules present in any one individual (Merkenschlager et al., 1997; Zerrahn et al., 1997). From these cells, however, only a small subset (3-5%) will be selected to mature (Huesmann et al., 1991; Shortman et al., 1991), which recognizes [self-peptide:MHC] complexes with low avidity. Following positive selection, a shut-down of one of the two co-receptors CD4 and CD8 occurs. The processes that lead T cell to commit to either the CD4 or CD8 lineages are still under debate. The body of existing data support an inductive/selective model of lineage commitment (Goldrath et al., 1997; Itano et al., 1996; Matechak et al., 1996; Sebzda et al., 1997), where both the avidity of the TCR[peptide:MHC] interaction together with influences from cell survival and fate genes will induce different flows of differentiation (Benoist and Mathis, 1998).

A great deal of work has been done in recent years to try to define precisely how immature thymocytes recognize self-peptides during the process of positive selection. Thanks to the use of in vitro fetal thymic organ culture systems from TCR transgenics and MHC class I processing mutants (β2-microglobulin or TAP knockout mice), where class I expression could be restored by
exogenously adding synthetic peptides back to the culture, it was possible to study the peptide requirements which allowed positive selection of immature thymocytes (Ashton-Rickardt et al., 1993; Hogquist et al., 1993; Sebzda et al., 1994). From those studies a differential avidity model for thymic selection was proposed, where low avidity interactions between the TCR and the [self-peptide:MHC I] complexes lead to positive selection, whereas high avidity interactions lead to negative selection of immature thymocytes (Ashton-Rickardt and Tonegawa, 1994). A stringent requirement for peptide recognition was first suggested by these studies, later supported by BIAcore™ measurements (Alam et al., 1996), where high affinity ligands for the TCR (agonists) lead to T cell deletion, while low affinity ligands (antagonists, partial agonists) lead to positive selection. For CD4+ T cell selection, peptide specificity was studied in vivo either by making use of mice engineered to express a single [peptide:MHC II] complex (Grubin et al., 1997; Ignatowicz et al., 1996; Ignatowicz et al., 1997; Surh et al., 1997; Tourne et al., 1997) or by identifying naturally expressed self-peptides, capable of mediating positive selection (Hogquist et al., 1997; Hu et al., 1997). The studies lead to three important findings. First, that multiple self-peptides have the capacity to contribute to selection of a single TCR (Hogquist et al., 1997; Hu et al., 1997). Second, that a single covalent [peptide:MHC II] complex could select 30-50% of the number of CD4+ T cells normally selected in wild-type mice, although displaying a different TCR repertoire (Grubin et al., 1997; Ignatowicz et al., 1996; Ignatowicz et al., 1997; Surh et al., 1997; Tourne et al., 1997). And third, that the selected CD4+ T cells could mount responses to a variety of peptides, including those bearing no sequence homology to the single selecting peptide (Grubin et al., 1997; Hogquist et al., 1997; Hu et al., 1997; Ignatowicz et al., 1997; Surh et al., 1997; Tourne et al., 1997).

These observations, in conjunction with other studies (Ashton-Rickardt et al., 1993; Nakano et al., 1997; Sebzda et al., 1994), have dismissed the notion that antagonist and agonist properties of the selecting ligand directly correlate with positive and negative selection, and more generally, that a similarity must exist between selecting and antigenic ligand. The promiscuity of selection demonstrated by these studies is probably the result of the same flexibility in ligand recognition by the TCR observed in mature, peripheral T cells (Evavold et al., 1995; Jameson et al., 1994; Kersh and Allen, 1996; Kersh and Allen, 1996; Sloan-Lancaster and Allen, 1996; Wucherpfennig and Strominger, 1995).
5.1. Positive selection in the periphery

Several recent reports have suggested that following emigration from the thymus, naive mature T cells require continuous TCR ligation by MHC molecules to survive in the periphery, a process similar to positive selection in the thymus. This was demonstrated for both CD8\(^+\) (Tanchot et al., 1997) and CD4\(^+\) T cells (Brocker, 1997; Kirberg et al., 1997; Rooke et al., 1997; Takeda et al., 1996). It was shown that monoclonal naive TCR-transgenic T cells require allele-specific interaction with MHC class I (for CD8\(^+\) T cells) and MHC class II (for CD4\(^+\) T cells) in order to survive in the periphery (Kirberg et al., 1997; Tanchot et al., 1997). Survival studies comparing naive versus memory CD8\(^+\) T cells suggested that memory CD8\(^+\) T cells also need to interact with MHC class I molecules to survive, although they may be less particular regarding MHC class I restriction (Tanchot et al., 1997).

6. T cell tolerance

As mentioned previously, the TCR recombination machinery can give rise to a nearly infinite repertoire of possible antigen-specificities. However, since the T cells are MHC-restricted in their antigen-recognition, only those specificities that are capable to recognize foreign-antigens in the context of the body's own MHC will be capable of contributing to adaptive immune responses. At the same time, T cells which recognize self-antigens in the context of self-MHC need to be rendered tolerant, in order to avoid an attack against the body's own tissue. The term immunologic tolerance comprises all the different mechanism through which autoimmunity is prevented. For the T cell compartment, the most radical mechanism is certainly the clonal deletion of self-reactive T cells during T cell selection in the thymus (negative selection). Other regulatory pathways, including the induction of clonal unresponsiveness (Nossal, 1983; Nossal and Pike, 1980; Ramsdell et al., 1989; Roberts et al., 1990) and immune deviation (Rocken and Shevach, 1996) also contribute to the general state of peripheral self-tolerance (McFarland, 1996).

6.1. Negative selection in the thymus

The possibility that self-reactive clones of T cells are deleted in the thymus was suggested many years ago (Lederberg, 1959) and has since been extensively demonstrated. The deletion of self-reactive cells during thymic education is now considered a principal mechanism through which self-tolerance is ensured in the T cell repertoire. Formal proof for clonal deletion came from a variety of studies with transgenic mice expressing TCR specific for self-antigen (Berg et al., 1989;
Blackman et al., 1990; Bluthmann et al., 1988; Kisielow et al., 1988; Murphy et al., 1990; Pircher et al., 1989; Sha et al., 1988; Zal et al., 1994) or mice endogenously expressing superantigens (Kappler et al., 1987; Kappler et al., 1988; MacDonald et al., 1988; MacDonald et al., 1988; Pircher et al., 1989), which showed that T cells with high affinity for self-antigens are deleted during T cell development. It is now clear, that negative selection by clonal deletion does not require a dedicated antigen-presenting cell (Iwabuchi et al., 1992; Pircher et al., 1993) and many stromal cells types were shown to induce clonal deletion. Particularly efficient in this role are the bone marrow-derived dendritic cells (DC) in the medulla (Kishimoto et al., 1996; Matzinger and Guerder, 1989; Sprent et al., 1988), but epithelial components as well as antigen carried on T cells themselves are also able to induce clonal deletion (Hoffmann et al., 1992; Oukka et al., 1996; Schonrich et al., 1993; Speiser et al., 1992). Deletion by negative selection can occur at various points along the differentiation pathway (Kappler et al., 1987; Zal et al., 1994). Factors which can influence the stage at which the deletion is induced are the nature of the cell presenting the deleting antigen, its anatomic location, and probably the overall avidity of TCR:\[peptide:MHC\] interaction (Hengartner et al., 1988; MacDonald and Lees, 1990; Pircher et al., 1989; Speiser et al., 1992).

One complication with selecting T cells on [self-peptide:MHC] complexes in the thymus, is that the same ligands are also expressed in the periphery. Different groups explained the fact that the T cell can be selected on a [self-peptide:MHC] ligand and yet tolerate it in the periphery by showing that immature thymocytes have a lower threshold of activation than peripheral T cells (Jameson et al., 1994; Pircher et al., 1991; Vasquez et al., 1994; Yagi and Janeway, 1990).

As a consequence of negative selection developing DP thymocytes will die in situ (Surh and Sprent, 1994) via programmed or activation-induced cell death (Jenkinson et al., 1989; Murphy et al., 1990; Robey and Fowlkes, 1994; Smith et al., 1989). It has been estimated that two thirds of the cells positively selected on [self-peptide: MHC] in the cortex will subsequently be deleted by negative selection through engagement of further [self-peptide:MHC] complexes on medullary cells (Huesmann et al., 1991; Ignatowicz et al., 1996; Merkenschlager et al., 1997; Shortman et al., 1991; Tourne et al., 1997; van Meerwijk et al., 1997).

A number of investigators have reported that clonal unresponsiveness (anergy) rather than clonal deletion can be induced during thymic selection (Blackman et al., 1990; Rammensee et al., 1989; Ramsdell et al., 1989; Roberts et al., 1990; Schonrich et al., 1992). Studies involving superantigens or TCR transgenic models suggested that the ligand density of the [self-peptide:MHC] complex and the maturation status of the thymocyte are determinant factors of the occurrence of anergy as opposed to deletion (Blackman et al., 1990; Ramsdell and Fowlkes, 1990; Ramsdell et al., 1989).
In summary, the body of available data suggest a model where the avidity of the interaction between the TCR and the [self-peptide:MHC] complex will determine whether an immature T cell will receive a signal for positive or negative selection. Different subsets of these thymocytes might be selected by [self-peptide:MHC] complexes existing at low density while other will be selected by [self-peptide:MHC] complexes present at higher ligand density. A possible prediction will be that the density of [self-peptide:MHC] complexes on which a T cell is selected might determine the relatedness between the selecting ligand on the cortical epithelium and the activating ligand in the periphery (Bevan, 1997; Kersh and Allen, 1996).

The induction of unresponsiveness (anergy) was shown by several investigators to occur after positive selection and is a further mechanism through which self-tolerance can be maintained.

6.2. Peripheral T cell tolerance

Although negative selection is very effective in deleting T cells binding with too high an avidity with [self-peptide:MHC] complexes, not all potentially autoreactive T cells are eliminated during T cell development (Kawai and Ohashi, 1995; Lo, 1992; Lo et al., 1988; Ramsdell et al., 1989; Roberts et al., 1990; Schonrich et al., 1991; Scott et al., 1994). In vitro studies as well as in vivo immune manipulations, which resulted in the development of autoimmune diseases have demonstrated that self-reactive T cells are responsible for the initiation of autoimmunity (Aichele et al., 1996; Heath et al., 1992; Mason and Fowell, 1992; Sakaguchi et al., 1985; Sinha et al., 1990; Sprent et al., 1990). Therefore, clonal deletion is not the only way the immune system protects the host from autogressive T cells. The induction of tolerance among T cells which have completed their development and migrated out of the thymus into peripheral lymphoid organs is referred to as “peripheral T cell tolerance”. Peripheral T cell tolerance can be induced because the self-antigens are presented to the T cells under conditions that do not allow effective immune responses to develop, or because the responses of the specific T cells are tightly regulated.

Some potentially self-reactive T cells which are exported to the periphery down-regulate the expression of their TCR or of their CD4/CD8 co-receptor molecules, reducing the avidity of interaction with [self-peptide:MHC] complexes expressed on peripheral tissues (Arnold et al., 1993; Ferber et al., 1994; Schonrich et al., 1991). Alternatively, the self-reactivity of other T cells was shown to be attenuated by the expression of a second endogenous TCRα chain (Heath and Miller, 1993). In other instances, the self-antigen to which positively selected T cells are reactive will never reach a lymphoid organ for long enough periods of time or at high enough concentration to ever activate the T cells. This phenomenon has been termed “immunological ignorance”, because
the T cells remain indifferent towards the self-peptides (Aichele et al., 1996; Hammerling et al., 1991; Lo et al., 1991; Zinkernagel, 1996; Zinkernagel et al., 1993). This is believed to be the case for most self-peptides located in sequestered sites (Barker and Billingham, 1977; Ohashi et al., 1991; Oldstone et al., 1991), or which are expressed at very low levels (Ferber et al., 1994; Ohashi et al., 1993). Tolerance in peripheral T cells may also be induced by a process of functional inactivation (anergy). In vitro, the induction of anergy has been demonstrated to be the result of antigen recognition in the absence of co-stimulation or IL-2 (Schwarz, 1990). The induction of clonal anergy in vivo has been best demonstrated upon administration of superantigen, where the deletion of CD4+ T cells is accompanied by the appearance of a residual population of T cells with the capacity to persist for some time in an anergic state, possibly because the avidity of binding to the TCR does not reach the threshold required for deletion (Kawabe and Ochi, 1990; Rammensee et al., 1989; Rellahan et al., 1990). However, the induction of anergy in vivo appears to be less due to a lack of co-stimulation than to the use of CTLA-4 to recognize the B7 molecules on the activated APC (Perez et al., 1997). In the study by Perez and colleagues, in fact, blocking CTLA-4 completely prevented tolerance induction (Perez et al., 1997), while in other studies, CD28 blockade lead to partial activation in the responding T cell, with a shift in the cytokine profile from Th1 to Th2 (Bluestone, 1995; Kearney et al., 1995). Alternatively, peripheral anergy could occur in the presence of co-stimulation upon TCR engagement with an endogenous altered peptide ligand (Sloan-Lancaster et al., 1993; Sloan-Lancaster et al., 1994). While APL-induced anergy has only been shown in vitro so far, the biological relevance of endogenous APLs in mediating peripheral tolerance has been demonstrated in vivo with the induction of antagonism (Basu et al., 1998; Vidal and Allen, 1996; Vidal et al., 1996; Williams et al., 1998).

The observation that mutation of the fas, fasL (Adachi et al., 1995; Nagata and Suda, 1995), CTLA-4 (Chambers et al., 1997; Tivol et al., 1995; Waterhouse et al., 1995), IL-2 or IL-2R genes (Sadlack et al., 1993; Suzuki et al., 1995; Willerford et al., 1995) in mice lead to various manifestations of autoimmunity while leaving central tolerance unaffected, suggested that Fas-FasL, CTLA-4 and IL-2 molecular interactions are involved in the maintenance of peripheral self-tolerance through regulatory mechanism which are triggered by antigen recognition. In other words, self-reactive T cells present in the periphery might actually be triggered upon encounter with self-antigens, but regulatory mechanisms will ascertain that the self-response will be aborted. The nature, dose, localization and persistence of the antigen are all factors which might determine whether CTLA-4 dependent anergy, Fas-mediated apoptosis or other mechanisms will abrogate the self-reactive response (Zinkernagel et al., 1997). For example, in a situation where the self-antigen is present all over the lymphoid system, like in the case of cell-associated and soluble self-antigens in the blood, self-tolerance will be achieved through the rapid induction of all self-reactive T cells,
which will be eliminated after a few days through activation-induced cell death (AICD), a phenomenon called "exhaustion".

Finally, a number of experimental models have been developed which suggest that peripheral tolerance may be maintained by regulatory/suppressor T cells (Asano et al., 1996; Fowell and Mason, 1993; Powrie et al., 1994; Sakaguchi et al., 1985; Sakaguchi et al., 1995; Saoudi et al., 1996), although this mechanism remains to be characterized. Different models have been proposed, including bystander suppression mediated by regulatory cytokines. In support to this model, multiple studies now suggest that downregulatory cytokines (TGF-β, IL-4 and IL-10), when produced by a bystander T cell population, can prevent autoimmune disease by damping down Th1, inflammatory-type immune responses (Bridoux et al., 1997; Chen et al., 1994; Fowell and Mason, 1993; Groux et al., 1997; Powrie et al., 1996; Weiner, 1997; Weiner et al., 1994).

7. T cell-mediated Autoimmunity

The pathophysiological consequence of the failure of the tolerance mechanisms mentioned above, is the development of an autoimmune response. Both genetic and environmental factors have been described to contribute to the breakdown of immunologic tolerance and the development of autoimmunity. Some of the factors relevant to the experimental part of this thesis are reviewed below.

7.1. Genetic predisposition

7.1.2 MHC and autoimmune disease association

The genetic predisposition to autoimmune disease was addressed by determining the disease prevalence in identical versus nonidentical twins and within families containing affected individuals compared with the prevalence in the general population (Blackwelder and Elston, 1985; Ebers et al., 1995; Vyse and Todd, 1996). From these studies, a number of genes have been described which contribute to the predisposition to autoimmune disease with the major influence being encoded within the major histocompatibility complex (MHC) (Leiter, 1989; Nepom, 1993; Nepom and Erlich, 1991; Nepom et al., 1987; Scharf et al., 1989; Sollid et al., 1989; Tiwari and Terasaki, 1985). The association between autoimmune disease (including rheumatoid arthritis, pemphigus vulgaris, multiple sclerosis, celiac disease and type I diabetes) and particular MHC
haplotypes has been known for over two decades, yet the mechanism of this association remains controversial. The difficulty in deciphering how MHC molecules function in autoimmune disease results from two complications: 1) MHC molecules play a critical role not only “restricting” T cell responses in the periphery, but also in shaping the T cell repertoire in the thymus. Hence the response or lack of response of a population of T cells to an antigen can be determined by either central repertoire selection or by the capacity for peripheral activation of T cells by [peptide:MHC] complexes. It is complicated to sort out whether the defect allowing autoimmune “forbidden” T cell responses in autoimmunity originates from the central or peripheral function of the MHC, or both. An attempt to unpuzzle this first complexity is presented Chapter IV of this thesis. 2) In autoimmune syndromes the MHC acts in conjunction with multiple other genes products to produce a polygenic, complex, autoimmune phenotype (Ghosh et al., 1993; Lander and Schork, 1994). Hence the MHC effect in autoimmune diseases occurs in a multi-gene immunologic pathway which is resistant to analysis; the weight of evidence suggests that the presence of disease susceptible MHC alleles, although permissive for disease, is required but not sufficient for disease to fully develop. The role of non-MHC-encoded genes in disease development has been best demonstrated in an animal model for autoimmune diabetes, the NOD mouse. By creating congenic and back-crossed strains, it became clear that the NOD MHC class II molecule put on other strains by itself was not sufficient for disease, and that several non-MHC genes on different chromosomes were also required (Ikegami and Makino, 1993; Leiter, 1989; Vyse and Todd, 1996; Wicker et al., 1993). Currently, except for the insulin gene (IDDM2) (Julier et al., 1991) and possibly the interleukin 2 gene (Idd3) (Denny et al., 1997) the identity of any non-MHC genes that predispose to autoimmune disease is not known. The recent availability of maps of markers that cover the entire mouse (Dietrich et al., 1996) and human genomes (Dib et al., 1996; Hudson et al., 1995) is now promoting the study of non-MHC genes that predispose to immunity.

7.2. Environmental influence

Genetic factors are known to contribute less than half of the life time risk of developing autoimmune diseases (Campbell et al., 1990). In addition to genetic predisposition, there is persuasive evidence that environmental processes are involved in the development of autoimmune responses.
7.2.1. Infection and autoimmune disease

The major environmental agents are probably infectious (Theofilopoulos, 1995). Several possible mechanisms have been proposed by which infectious agents could trigger, promote or maintain autoimmune disease. Some of these mechanisms are reviewed below.

**Induction of antigen processing/presentation and costimulatory molecules**

The processing and presentation of cryptic determinants of self-proteins can be induced under inflammatory conditions (Kaufman et al., 1993; Lehmann et al., 1992; Lehmann et al., 1993; Opdenakker and Van Damme, 1994; Tisch et al., 1993) and recruits T cells which might have escaped tolerance induction in the thymus (Gammon and Sercarz, 1989). It is conceivable that infection of autoimmune target tissues, by causing an inflammatory reaction, might lead to the availability of normally sequestered antigen for uptake, processing and presentation by local APCs. In the absence of inflammation, the display of previously cryptic self-determinants has also been observed upon downregulation of the CD4 molecule by gp120 protein of human immunodeficiency virus (HIV) (Salemi et al., 1995). Infection products of local inflammation like IFN-γ and TNF were shown to up-regulate the expression of MHC molecules, costimulation and adhesion molecules on local APCs (Campbell et al., 1989; Campbell et al., 1988; Green et al., 1998; Neufeld et al., 1989; Parkkonen et al., 1992). Therefore, up-regulated presentation of normally expressed but normally ignored self-determinants in the context of appropriate costimulation on local APCs could induce immunity by providing the second signal required to the activation of self-reactive T lymphocytes that had remained “ignorant”. Alternatively, self-antigens that might be released by cells destroyed by cytopathic infections could be transported into the lymphoid organs and presented by professional APCs to self-reactive T cells (Aichele et al., 1996; Zinkernagel et al., 1997).

**Molecular mimicry**

Molecular mimicry has received a lot of attention as a potential mechanism for initiating and sustaining autoimmune disease. Infectious agents are predicted to carry antigenic determinants which resemble self-determinants, such that an activated pathogen-specific lymphocytes might cross-react with the host’s self (Fujinami and Oldstone, 1985). Although molecular mimicry between bacterial and viral protein and endogenous molecules has been suggested to play a role in both human diseases (Hausmann and Wucherpfennig, 1997) and animal models (Tung et al.,
1997), currently only few examples exist where molecular mimicry was shown to be the initiating factor of an autoimmune disease (Bachmaier et al., 1999; Evans et al., 1996; Fujinami and Oldstone, 1985; Ohashi et al., 1993; Wucherpfennig and Strominger, 1995). Most speculations about molecular mimicry acting as a mechanism in the induction of autoimmunity are to be attributed to computer-generated matches between known microbial epitopes and autoantigens and are not yet validated experimentally.

**Modification of Th1/Th2 balance**

The evidence that T cells are functionally heterogeneous, as a result of their different cytokine profiles (Abbas et al., 1996; Mosmann et al., 1986; Mosmann and Coffman, 1989) has offered an explanation for the ability of certain T cells to induce autoimmunity. Evidence accumulating from experimental models and human pathological conditions suggest that the relative contribution of either Th1 or Th2-dominated reactions can determine the development of a particular autoimmune response. In chronic autoimmune diseases, such as multiple sclerosis and insulin-dependent diabetes mellitus, pathogenic roles have been attributed to Th1 cells (Liblau et al., 1995; O'Garra and Murphy, 1993; O'Garra et al., 1997; Powrie and Coffman, 1993). Since cytokines produced by Th2 cells can down-regulate the development of Th1 driven cell-mediated immune responses (Abbas et al., 1996; O'Garra and Murphy, 1994; Paul and Seder, 1994), a protective role has been attributed to Th2 cells in the development of organ-specific autoimmunity. Recently the ability of inhibiting initiating or ongoing autoimmunity has been extended to further regulatory populations, named Th3 and Tr1 which produce TGF-β in addition of some Th2-like cytokines (Bridoux et al., 1997; Chen et al., 1994; Groux et al., 1997; Powrie et al., 1996; Powrie and Mason, 1990; Weiner, 1997). Conceivably, any modification of the general Th1/Th2 immune balance through genetic or environmental factors might bias a response towards immunity or tolerance. Upon infection with intracellular pathogens, macrophages will be stimulated to produce IL-12 and IFN-α (which in turn will induce IFN-γ production by both T cells and NK cells) promoting Th1 differentiation of the immune response (Charlton and Lafferty, 1995; Romagnani, 1992; Sher and Coffman, 1992). Even though Th1 responses are appropriate for the eradication of microbial pathogens, if elicited inappropriately against self-antigens, as in the context of molecular mimicry, they could lead to tissue destruction and pathology (Bachmaier et al., 1999; Liblau et al., 1995; O'Garra et al., 1997). The transgenic overexpression of the cytokines IFN-γ or -α in pancreatic islets of non-susceptible strains of mice provided a direct demonstration of the critical role of pro-inflammatory cytokines in determining the loss of tolerance to self antigens within target tissues: IFN-γ and IFN-α transgenic mice developed massive infiltration of inflammatory...
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cells and total destruction of insulin-secreting cells resulting in diabetes (Sarvetnick et al., 1988; Sarvetnick et al., 1990; Stewart et al., 1993).

7.2.2. Non-infectious influence

Some of the non-infectious, environmental factors which were discovered to promote autoimmunity include ultraviolet radiation, which may expose intracellular epitopes through apoptosis, environmental pollutants, which may induce changes in nucleolar constituents, medicinal drugs, which can induce lupus by promoting hypomethylation of DNA and anesthetic agents like halothane that create novel antigens in the liver (Rose and MacKay, 1998).
8. Aims of the study

The frequency of antigen specific T cells can be on the order of 1:50,000-100,000 in an unprimed mouse and approximately 1:1000-10,000 in primed individuals, which makes their analysis extremely difficult. Therefore, the study of Th cell development in vivo has mostly relied on the adoptive transfers of fixed-TCR transgenic T cells into antigen-immunized recipient mice. However, this approach not only involves the manipulation of the normal physiology of the immune response, but also fixes the repertoire, preventing the analysis of TCR repertoire development and clonal maturation. Multiparameter flow cytometry as a method to visualize T cell responses in normal animals, has provided new information on clonal maturation of the Th-response in vivo. This technique relies on the combination of a clonotypic antibody against the dominant TCR along with antibodies against markers of activation/memory. While this method is used to identify the antigen-specific T cell population to a particular antigen in vivo, it is still limited to those responses which use dominant TCR regions.

The discovery that upon antigen challenge antigen-specific T helper cells up-regulate cell surface expression of CD4 is reported in Chapter I of this thesis. The first aim of the study was to use the DBA/2 response to the antigen Sperm Whale Myoglobin (SWM) amino acids 110-121 in order determine whether CD4 up-regulation uniquely characterizes a population of recently activated antigen-specific T cells both in vitro and in vivo, and to define its potential application in the study of Th cell response development in vivo.

Having shown the relevance of CD4 as a marker for T cell activation, the question of whether T cells, similar to B cells, could undergo a process of affinity selection during the course of the immune response in vivo, was addressed in Chapter II. A defining feature of the adaptive immune response is its capacity to “remember” a previous antigenic challenge, such that upon re-exposure with the same antigen, a faster and stronger immune response occurs. While development of memory in the B cell compartment appears to be based on increased frequency, isotype switching and affinity maturation of antigen-specific antibodies, the mechanisms underlying T cell memory are still obscure. Since CD4 up-regulation could be used to identify and isolate the entire antigen specific population in response to SWM 110-121 in vivo, the aim of this study was to establish a system allowing to investigate both phenotypical and functional changes during an antigen-specific T cell response in vivo, thus shedding light upon the development of immunologic memory.

Upon activation, CD4+ T cells undergo differentiation into effector T cells releasing characteristic patterns of cytokines. The type of cytokines present at the initiation of the immune
response, including IL-12 and IFN-γ, or IL-4 play a central role in controlling the development of CD4 T cells into distinct Th subsets. In addition, the avidity of the TCR:[peptide:MHC] interaction directly affects lineage commitment of CD4+ T cells. The avidity model is mostly based on in vitro experimental findings, where changes in peptide structure and density (through use of peptides mutated in MHC or TCR binding sites) can directly affect the development of TCR transgenic T cells towards Th1 or Th2 development. It is difficult, however, to draw similar conclusions from in vivo immunization experiments. Due to the difficulty of controlling all three variables of the trimeric TCR:[peptide:MHC] interaction in vivo, the T cell developmental effects observed in in vivo immunization models can not be easily correlated to variations in the overall avidity of the TCR:[peptide:MHC] interaction. The “CD4hi-LDA cloning technique” presented in Chapter II offered an alternative approach: in our system, the SWM dose and MHC expression were kept constant and only the T cell affinity for the [peptide:MHC] complex was allowed to vary. Therefore, the first aim of the study presented in Chapter III was to address the role of the affinity of the TCR in the population of responding T cells for the [peptide:MHC] complex in the development of a Th1 or Th2 response in vivo using our “CD4hi-LDA cloning technique”. The second aim was to define the role of the overall affinity of the pre-existing T cell repertoire in affecting the Th1/Th2-lineage commitment of responses against self versus foreign antigens.

The examination of self versus foreign-specific T cell response development was extended to an autoimmune model of disease, the NOD mouse. Chapter IV of this thesis summarizes these findings, as well as an alternative explanation for the role of MHC in autoimmune disease. The prevailing paradigm of MHC and disease association is presentation of autoantigens to the immune system by disease associated MHC molecules, leading to an immune response and resultant autoimmune sequelae. The finding of a structural difference between disease-associated and nonassociated MHC haplotypes in diabetes initially appeared to strongly support this theory. However, the finding of multiple autoantigens in NOD mice, linked to a variety of immune syndromes in addition to diabetes, and the observation that the genetics of MHC association were not recessive, but dominant with a very poor penetrance, raised significant problems for the dominant theory of the association. The new model for MHC and disease association presented in Chapter IV seeks to resolve these conflicting observations.
Figure 1. Antigen-specific recognition by B and T cell receptors

A) Crystal structure of two antigen binding fragments (Fab) of a B cell receptor, complexed with the antigen phencyclidine (PCP). PCP is shown in solid images in the binding site of each Fab fragment.
(From Lim et al., 1998)

B) Crystal structure of a TCR:[peptide:MHC] complex
(from Davis et al., 1998, adapted from Garcia et al., 1996).
Figure 2. Kinetic proof reading model for T cell activation.

a) TCR ligation by immunogenic ligand leads to efficient recruitment and/or activation of the src kinases, saturated phosphorylation of all CD3 immunoreceptor tyrosine-based activation motives (ITAMs) (filled circles), and full T cell activation.

b) Due to low affinity, short-lived interaction, APL engagement by the TCR causes inefficient recruitment and/or activation of src kinases, and incomplete phosphorylation of CD3 ITAMs, resulting in a lack of binding templates for some SH2-domain containing proteins. Thus, some signaling pathways remain inactive (arrow with open circles), other are stimulated weakly (arrow with open and filled circles) while others are activated normally (arrow with filled circles), directly dependent on the tyrosine state of the ITAMS, itself dependent on the length of TCR-ligand interaction.

(adapted from Sloan-Lancaster and Allen, 1996)
Figure 3. Serial triggering model for T cell activation.

A single [peptide:MHC] complex can engage and trigger many TCRs during the prolonged T cell-APC interaction. Engagement of a TCR by a single [peptide:MHC] complex for a sufficient time results in the phosphorylation of CD3/ζ ITAMs and recruitment of signal transduction components, triggering the TCR (indicated by an asterisk). The triggered TCR dissociates and the [peptide:MHC] ligand is free to engage and trigger another TCR. This dissociation is promoted by the high off-rate of binding and possibly by physical shearing stress due to T cell motility. The triggered TCRs signals for a short period of time and are subsequently removed by down-regulation.

Sustaining the process requires a continuous supply of signal transduction components from the cytosol, as well as new TCRs to substitute for those that are down-regulated and degraded. Cell-cell adhesion is boosted by TCR signaling and favors TCR engagement with [peptide:MHC] complexes.

(from Valitutti and Lanzavecchia, 1997)
Figure 4. Relevance of the serial triggering model for agonism and antagonism.

Each line represents the serial interactions of a single [peptide:MHC] complex with TCRs on the surface of the opposing T cell. The duration of the interaction is depicted as a solid black line, and the consequences of the interaction as a filled dot. When the interaction with the TCR is stable above a given threshold, the TCR is triggered (black dot). Lower affinity interactions may lead to partial activation (black/grey dot) inactivation (grey dot), or no effect at all (no dot).

A) An optimal agonist (marked with an asterisk) has an optimized affinity that allows both efficient triggering and recycling. Complexes with lower affinity than optimal agonists may serially engage more TCRs, but the outcome of each interaction may vary depending on the time of association, leading to different proportions of fully triggered, partially triggered, or inactivated TCRs. It is important to underline that at intermediate affinities the same [peptide:MHC] complex might in some cases trigger, and in others partially trigger or inactivate, the bound TCR, depending on the duration of each individual interaction. In all cases the cell will integrate the triggering signals up to a threshold when it becomes committed to proliferation and cytokine production.

B) TCR antagonism may be explained by sequential inactivation of many TCRs by low-affinity ligands. When agonists and antagonists are present on the same APC, the antagonists may rapidly spoil a large number of TCRs, because they recycle very effectively or because they are present in higher amounts. In this example, four antagonists rapidly inactivate many TCRs, thus reducing the pool of TCRs available for serial triggering by the agonistic complex.

(from Valitutti and Lanzavecchia, 1995).
CHAPTER I

Following Antigen Challenge, T Cells Up-Regulate Cell Surface Expression of CD4 In Vitro and In Vivo

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Grant Support

This work was sponsored by the National Institutes of Health grants DK39959 and DK44837. William Ridgway was supported by the Howard Hughes Medical Institute.

SUMMARY

The low precursor frequency of antigen-specific T cells has raised significant barriers to studying the T cell response \textit{in vivo}. We demonstrate that T cells, following antigen recognition, upregulate cell surface expression of CD4, which identifies antigen-specific T cells \textit{in vitro} and \textit{in vivo} and allows their characterization. The CD4\textsuperscript{high} cell subpopulation contains the antigen-specific population as shown by antigen-induced proliferation and limiting dilution analyses. The use of the CD4\textsuperscript{high} marker will allow analysis of the dynamics of the T cell immune response \textit{in vivo}, the study of suboptimal T cell response to antigen, and identification of T cells reactive to known and unknown autoantigens.
INTRODUCTION

The immune response to foreign protein antigens is dependent on antigen-specific CD4+ T cells. The frequency of T cells specific for any particular antigen, however, is extremely low, on the order of 1:50,000-300,000 T cells in unprimed, and approximately 1:1000-10,000 in primed individuals (Ford and Burger, 1983; Gebel et al., 1983; Kojima et al., 1988; Weyand et al., 1986), which makes their analysis extremely difficult. Although several methods have been developed to study T cell responses in vivo (e.g. adoptive transfer of T cell clones and TCR transgenic T cells) (Kearney et al., 1994; Kearney et al., 1995; Livingstone and Fathman, 1987), and some T cell responses to particular peptide antigens (e.g. peptides from pigeon cytochrome c) use highly restricted TCRs, enabling tracking of antigen responses with clonotypic antibodies (Altman et al., 1996; Davis et al., 1995; McHeyzer-Williams et al., 1996; McHeyzer-Williams et al., 1996; McHeyzer-Williams and Davis, 1995); the characterization of antigen-specific T cell responses requires manipulation of the normal physiology of the immune response. Thus, although these methods have provided important insights into T cell responses in vivo, they share some drawbacks in studies of conventional immune response.

T cell clones and T cells from TCR transgenic mice utilize a single TCR αβ heterodimer and these T cells generate a homogenous, single affinity response to antigen. The methods used to isolate T cell clones, and also the T cells from which TCR transgenic mice are derived, have used culture conditions predisposed to selecting only the best growing T cells from culture; the T cells with the proliferative response “most favorable” for growth. Monoclonal antibodies to clonotypic TCR alpha and beta chains can be used to study T cell immune responses in vivo, but only to those antigens which are known to elicit a clonotypic response; moreover the antibodies will only identify T cells with the clonotype discovered in vitro, thereby confounding the question of whether the T cell response in vivo is similar to the T cell response in vitro.

We have identified a novel way to investigate the response of antigen-specific T cells in vitro and in vivo which avoids these drawbacks. Here we demonstrate a model system, utilizing the DBA/2 response to SWM 110-121, to study antigen-specific T cells in vitro and in vivo. Our data demonstrate that antigen-specific murine T cells upregulate their surface expression of the CD4 molecule following antigen recognition in vitro and in vivo. The CD4^high cells contain all of the antigen-specific T cell proliferative response in vivo and in vitro. The CD4^high population also expresses memory/activation markers consistent with recently activated cells. The finding that antigen-specific T cells upregulate CD4 expression should aid studies on the dynamics and heterogeneity of the T cell immune response in vivo, the study of suboptimal or heterogenous immune responses, and the identification of T cells reactive to known and unknown autoantigens.
MATERIAL AND METHODS

Mice

DBA/2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the Stanford Medical Center Department of Comparative Medicine. Mice were used between 8 and 30 weeks of age.

Antigen Proliferation assays

Groups of 3-5 mice were immunized at the base of the tail with 100 µl of an emulsion containing IFA plus 10 mg/ml heat killed Mycobacterium tuberculosis, H37RA (Difco Laboratories Inc, Detroit MI) plus 100 µg peptide suspended in an equal volume of Dulbecco's PBS. The peptide sperm whale myoglobin (SWM) 110-121 (AIIHVLHSRHPG) was synthesized and HPLC purified at the Protein and Nucleic Acid Facility, Beckman Center, at Stanford University (Stanford, CA). 8-10 days after immunization, draining inguinal lymph node cells were removed and single cell suspensions prepared. For whole lymph node proliferation assays 5 x 10^5 cells were incubated in 96 well flat bottom plates in either T cell media alone or with antigen. T cell media consisted of RPMI 1640 supplemented with 2 mM L-Glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and 10 mM hepes buffer (Gibco Laboratories, Grand Island, NY), 50 mM 2-ME (Sigma Chemical Co., St. Louis MO), and either 0.5% normal mouse serum or 5-10% FCS. After 72 h at 37 degrees C, 6% CO2, cells were pulsed with 1 µCi of [3H] thymidine and harvested 18 hours later for counting on a Beta plate (Wallac, Inc., Gathersburg, MD).

FACS analysis

For studies in vitro, aliquots of lymph node cells were taken from culture, washed with FACS buffer (Dulbecco's PBS with 2% FCS), stained for 2 color flow cytometric analysis with fluorescein and phycoerythrin antibodies at a predetermined optimal concentration for 20 minutes at 4 degrees C, washed, and stained with propidium iodide (Sigma, St. Louis, MO) just prior to analysis. Anti-CD45RB, anti-CD44, anti-CD69 and anti-Vß8 fluoresceinated antibodies were obtained from Pharmingen, Inc. (San Diego, CA ), as was anti-Mel-14 biotinylated antibody. Anti-CD4 and strepavidin—phycoerythrin antibodies were obtained from Caltag (South San Francisco, CA ). 1x10^5 cells were analyzed by two color flow cytometry on a Becton—Dickinson Faccscan cytometer. The data was analyzed using the Herzenberg desk facility plus Flowjo (Tree Star, Inc., San Carlos, CA) on a Power Macintosh. Statistical analysis including the two sided T test was
performed with Statview (Abacus Concepts, Inc., Berkeley CA,) and Excel (Microsoft, Redmond, Washington). Propidium iodide positive cells were excluded from analysis.

**FACS sorting and proliferation assays**

Single cell suspensions were obtained either from 3 day cultures or directly from the lymph nodes of immunized animals (in both cases, harvested 8-10 days after immunization, as above). The cells were stained with anti-CD4 antibody (and in some experiments also with anti-Vβ8 antibody). The cultured cells were analyzed by FACS for determination of appropriate gates, sterile sorted into positive and negative populations, plated into 96 well plates and pulsed with $[^3H]$ thymidine for 18 hours, then counted on a Beta plate. Cells sorted directly from immunized lymph nodes were analyzed, and the highest 1% of cells were selected for sorting; generally 1% analysis levels resulted in actual sorting of the top ~5% of cells. The cells were cultured in 96 well plates at varying numbers (generally between $1-2 \times 10^4$) of CD4$^{high}$ versus CD4$^{normal}$ cells, or alternately CD4$^{high}$ Vβ8$^+$ versus CD4$^{normal}$Vβ8$^+$ cells; in 96 well plates containing $5 \times 10^5$ irradiated syngeneic lymph node or spleen cells with or without antigen. The cells were then cultured 72 hours before pulsing and harvesting as above.

**Limiting dilution analysis**

Cells from either culture or directly from immunized mice, were FACS sorted as above. After sorting, small aliquots of positive and negative populations were resuspended in FACS buffer and analyzed on the FACS machine used for sorting to assess for percent purity of the sorted populations. The sorted cells were then pelleted and titrated at varying cell numbers into 96 well plates containing irradiated DBA/2 spleen, 10 μM SWM 110-121, and 10 units/ml IL-2. The plates were cultured for 10 days, at which time the cells were transferred into fresh 96 well plates containing irradiated DBA/2 spleen with or without SWM110-121, without IL-2. The cells were cultured an additional 3 days, then pulsed with 1 μCi/well $[^3H]$ thymidine, and harvested for counting 18 hours later. The proliferative response was analyzed for responders/nonresponders using 4 standard deviations above the response in absence of antigen as a cutoff for positive responders. Cells were obtained for analysis without antigen in two ways: either some wells in the replicate wells were cultured without antigen, or some wells were split at the time of restimulation and cultured with or without antigen. The proliferative response of CD4$^{high}$ cells cultured without antigen was consistent across experiments. In some experiments, duplicate plates were cultured at the time of restimulation; one plate used for proliferative response while the second plate was maintained in culture. The percent negative wells was plotted against cells/plate and analyzed by least squares linearization using Cricket Graph (Computer Associates, Inc.), the exponential curve
Chapter 1 T cells up-regulate CD4 in response to their nominal antigen

fitting function produced an equation of the form \( y = (\alpha) x 10^{(\beta x)} \); \( \alpha \) and \( \beta \) were derived from the data and the resultant precursor frequency (x) was calculated by setting \( y = .37 \) according to Poisson statistics.

RESULTS

Antigen-specific T cells are CD4^{high}

We initially used the DBA/2 response to SWM 110-121, previously characterized in our lab (Danska et al., 1990; Ruberti et al., 1991; Ruberti et al., 1993; Weyand et al., 1986), to analyze antigen-reactive T cells. Groups of mice were immunized with peptide, immune lymph node cells were harvested 8-10 days later, and cultured with or without the immunizing antigen. Periodically, aliquots of cells were taken from culture, stained with antibodies to cell surface molecules, and analyzed by flow cytometry for kinetics of expression by live (PI negative) cells (Fig. 1). At 48 hours, and more obviously at 72 and 96 hours, a subpopulation of cells demonstrated increased CD4 expression (Fig. 1). These CD4^{high} cells were also CD45RB^{low}, CD62L^{low}, CD69^{high}, and CD44^{high} (Fig. 1). T cells from immunized lymph nodes cultured for similar periods of time without antigen showed no comparable CD4^{high} subpopulation (not shown). The percentage of CD4^{high} cells increased steadily throughout the culture period, from less than .4% of live cells at time zero to 10-20% of cells by 96 hours (Fig. 1). The increased percentage was due both to death of other cells in the culture over time, and to an absolute increase in the CD4^{high} cell numbers (data not shown). Statistical analysis of CD4^{high} levels at 72 hours after immunization (the usual time of pulsing in proliferation assays) of 13 separate experiments showed that the difference in CD4 expression between CD4^{high} and CD4^{normal} was highly significant (unpaired T test, \( p < .00001 \)). While there was some variance in the absolute level of CD4 expression in the experiments, the ratio of the CD4^{high} to CD4^{normal} was remarkably consistent over all experiments at 3.5:1. The mean ratios of CD45RB and CD44 in the CD4^{high} population were also significantly different from the CD4^{normal} population. The CD45RB expression in the CD4^{high} group was approximately 1/2 of the CD4^{normal} group. The CD4^{normal} population showed two subpopulations, one of which expressed ~165 fold less CD44 than the CD4^{high} cells, and a second population expressing ~3 fold less CD44 than the CD4^{high} population (Fig. 1).

The finding that the CD4^{high} cells in culture expressed memory/activation markers suggested that they might be the antigen-reactive T cells. To test this, we FACS sorted the CD4^{high} and CD4^{normal} cells after 72 hours in culture, using the distinct populations seen on FACS analysis (Fig. 1) to establish sorting gates. When the cells were sorted and pulsed with [^3]H thymidine, the
CD4\textsuperscript{high} population contained all the proliferating cells from the whole lymph node preparation, while the CD4\textsuperscript{normal} cells did not proliferate above background (Fig. 2B). Whole lymph node cell response to antigen from the same culture was used as a control (Fig. 2A). Thus, all of the antigen-specific proliferative response seen \textit{in vitro} in response to SWM 110-121 was contained in the CD4\textsuperscript{high} cell population.

\textit{Limiting dilution analysis of CD4\textsuperscript{high} T cells in vitro}

It was possible that only a small percentage of the CD4\textsuperscript{high} cells was actually proliferating in the sorting experiment shown in Fig. 2. Thus, we used limiting dilution analysis to quantify the enrichment of antigen reactivity in the CD4\textsuperscript{high} population. SWM immune DBA/2 lymph node cells were cultured with antigen for three days and sorted into CD4\textsuperscript{high} and CD4\textsuperscript{normal} subpopulations, then plated at titrated numbers onto irradiated DBA/2 spleen cells in the presence of antigen and IL-2. After 10 days culture, the cells were transferred to plates with fresh antigen and APCs, without IL-2, and pulsed for proliferative response after another 3 days culture. The fraction of negative wells was calculated, allowing determination of antigen-specific T cell frequency according to Poisson statistics (Fig. 3). The results presented in Fig. 3 demonstrate that the CD4\textsuperscript{high} subpopulation from culture contained a substantial enrichment of the antigen responsive cells; 1 in 5.1 CD4\textsuperscript{high} cells were antigen-reactive, compared to typical estimates of antigen frequency in immunized lymph nodes from 1 in 1000 to 1 in 10,000 whole lymph node cells (Ford and Burger, 1983; Gebel et al., 1983; Kojima et al., 1988), and compared to the estimate of whole SWM reactive T cells in primed DBA/2 mice of 1/11,625 previously published by our lab (Weyand et al., 1986). The mean of 5 LDA experiments was 1 in 8.2 CD4\textsuperscript{high} cells as antigen-specific. While the CD4\textsuperscript{normal} curve was not entirely horizontal (Fig. 3), the CD4\textsuperscript{normal} cells showed no antigen reactivity either after pulsing directly after harvesting from the immunized lymph node (Fig. 2B) or after restimulation with antigen for 3 days prior to pulsing (Fig. 4). Post sort analysis established that the residual reactivity seen in the LDA experiments was due to a minor population (< 1.5%) of CD4\textsuperscript{high} cells which contaminated the CD4\textsuperscript{normal} group during FACS sorting (data not shown).

\textit{Antigen-specific CD4\textsuperscript{high} T cells are present in immune lymph node cells in vivo}

These results demonstrated that the CD4\textsuperscript{high} cells contained the antigen responsive cells \textit{in vitro}. The adaptation of this method to studies \textit{in vivo} was then attempted. FACS analysis of freshly harvested lymph node cells from DBA/2 mice (taken at various time points after immunization with SWM 110-121) showed no obvious expansion of CD4\textsuperscript{high} cells (see Fig. 5A, top panel, compared to Fig. 1). We reasoned that the antigen-reactive CD4\textsuperscript{high} T cell population was present but had not expanded as dramatically \textit{in vivo} as \textit{in vitro}. To test this hypothesis we
again used the DBA/2 response to SWM110-121, which shows a predominant Vβ8 response when cells are cloned and T cell hybridomas are made (Danska et al., 1990; Ruberti et al., 1991; Ruberti et al., 1993). We sorted CD4<sup>high</sup> Vβ8<sup>+</sup> T cells directly from the lymph nodes of SWM immunized DBA/2 mice, using gates set to select the highest CD4 expressing Vβ8<sup>+</sup>T cells (representing ~0.5% of lymph node cells), and cultured those cells, versus CD4<sup>normal</sup> Vβ8<sup>+</sup> positive T cells, with naive irradiated DBA/2 spleen cells with or without antigen (the lymph node T cell proliferative response from the same mice served as control) (Fig. 4). The CD4<sup>high</sup> Vβ8<sup>+</sup> T cells demonstrated a robust antigen proliferative response, while the CD4<sup>normal</sup> Vβ8<sup>+</sup> T cells did not. These experiments demonstrated that the antigen-reactive T cell CD4<sup>high</sup> subpopulation could be identified in vivo.

**Limiting dilution analysis of CD4<sup>high</sup> T cells activated in vivo**

Next, we quantified the enrichment of the CD4<sup>high</sup> antigen-specific T cell precursor frequency of CD4<sup>high</sup> T cells activated in vivo using limiting dilution analysis. We sorted CD4<sup>high</sup> Vβ8<sup>+</sup> T cells directly from freshly harvested SWM 110-121 immunized lymph nodes 8 days after immunization (Fig. 5A), using the top ~1% of CD4 brightness in the CD4<sup>+</sup> Vβ8<sup>+</sup> cell population as a sorting gate (Fig. 5A). We cultured titrated sorted cell numbers in 96 well plates with naive irradiated DBA/2 spleen cells, antigen, and IL-2, and restimulated the cultures at 10 days. We consistently found a substantial enrichment of antigen reactivity in the Vβ8<sup>+</sup> response (1 in 32.4 CD4<sup>high</sup> cells, Fig. 5b) in the 8d in vivo SWM110-121 response. The result was quite consistent over 5 LDA experiments with a mean of 1/32.8 CD4<sup>high</sup> cells as antigen-specific from primed lymph nodes in vivo.

The frequency of CD4<sup>high</sup> Vβ8<sup>+</sup> cells for SWM110-121 in vivo was lower than in the analysis in vitro (Fig. 3 above). The lower precursor frequency demonstrated in vivo compared to studies in vitro could be partially attributed to the technical difficulty of extracting cells directly from the mouse and sorting/culturing them for prolonged periods. In addition, analysis of the response of the CD4<sup>high</sup> T cells to PPD demonstrated that some CD4<sup>high</sup> cells, when taken directly from immunized mice, were primed by PPD contained in the CFA used as adjuvant (data not presented). (CFA primed cells do not expand during culture with SWM, hence the CD4<sup>high</sup> population in vitro is further enriched for SWM reactive cells at the time of cell sorting from culture). While the CFA primed cells do not expand during restimulation in vitro with SWM, they will dilute the frequency of CD4<sup>high</sup> cells which recognize SWM at the time of sorting (sorting is done prior to expansion with the cells primed in vivo). A final explanation of the 4 fold less enrichment in vivo compared to in vitro sorting of CD4<sup>high</sup> is suggested by a comparison of the empiric sort gates chosen in vivo (Fig. 5A) compared to the gates established in vitro (Fig. 1).
consistent mean ratio of CD4\textsuperscript{high} to CD4\textsuperscript{normal} expression \textit{in vitro} suggested that the gates chosen \textit{in vivo} allowed a significant number of CD4\textsuperscript{normal} cells into the “CD4\textsuperscript{high}” population.

We have demonstrated that T cells from all mouse strains studied to date (following immunization with peptides or proteins) upregulate CD4 expression in response to antigen, e.g. (PLJ x SJL)\textsuperscript{F1} mice in response to MBP (Mike Shaw, personal communication), NOD mice in response to self and foreign peptides, and naive HEL 46-61 responsive transgenic T cells in culture (unpublished data). We postulate that upregulation of CD4 in response to peptide antigens is a universal T cell response to initial confrontation with “nominal” antigen \textit{in vivo}.

\textbf{CD4\textsuperscript{high} T cells contain a heterogenous population of responding T cells}

We next investigated whether CD4\textsuperscript{high} expression could be used as a method of identifying and characterizing antigen-specific cells \textit{in vivo} which are not normally expanded under competitive culture conditions (i.e. sub-optimal T cell proliferative responses). The CD4\textsuperscript{high} approach allowed the possibility of individually expanding sub-optimally reactive T cells directly from the immunized lymph node. To accomplish this, limiting dilution analyses were carried out as above, and, at the time of restimulation, CD4\textsuperscript{high}V\text{ß8}\textsuperscript{+} cells were plated into replicate plates. One of the two plates was then cultured for three additional days and pulsed with \textsuperscript{3H}thymidine to establish the dilution of cells at which one cell or less was present in the well. The proliferative responses of individual positive wells grown out from initial conditions at approximately one cell/well (Fig. 6) (as calculated by the LDA from Poisson statistics) was plotted. The responses of positive wells expanded under identical culture conditions from individual CD4\textsuperscript{high}V\text{ß8}\textsuperscript{+} cells showed a continuous distribution (over ~3 logs of CPM) of proliferative responses from very high responders to very low responders (Fig. 6). This distribution stands in contrast to the expected behavior of a clonal population, which should show a normal distribution of proliferative responses (i.e. most of the response occurring near a mean, with some outliers on each end). The uncloned CD4\textsuperscript{high} cells from immune lymph nodes, on the contrary, show equal numbers of cells proliferating at every level (Fig. 6). It is natural to speculate that if the fast growing CD4\textsuperscript{high} cells (at the far right as plotted in Fig. 6) were cultured under standard cloning conditions with slow growing CD4\textsuperscript{high} T cells (at the left in Fig. 6), the fast ones would outgrow the slower ones. The advantage of this limiting dilution method of cloning cells directly from the lymph node is that the separate cells can be expanded without the pressure of competitive growth, when cultured at one cell (or less) per well, thus enabling study of suboptimal responders to antigen \textit{in vivo}. There are two likely explanations for unequal growth of responding CD4\textsuperscript{high} cells plated under identical conditions. One is that an unknown stimulus, continuously distributed under the conditions of culture, causes T cells with inherently equal antigen responsiveness to proliferate unequally. The
second possibility is that the responding T cells display a continuous distribution of growth (proliferation) in response to a single antigen concentration. If the growth response reflects an intrinsic difference between the responding T cells, such as TCR affinity for antigen, the phenotype of low versus high responder should persist after repetitive stimulation and rest of the T cells. The system outlined here allowed us to investigate that question. Preliminary results indicate that low responder cells can be cloned (from the replicate plates) and maintained in culture, and not only retain their low proliferative phenotype, but express different (non-dominant) Vβ repertoires from the high responder cells (M. Fasso, manuscript in preparation).

**DISCUSSION**

Results presented here demonstrate that the antigen-specific T cells responding to an antigenic challenge in vitro and in vivo are the CD4$^{\text{high}}$ T cells. The CD4$^{\text{high}}$ population contains all the T cell proliferative response to antigen in vitro and in vivo (Figs. 2B and 4). Moreover, depletion of the CD4$^{\text{high}}$ T cells from whole lymph node populations which are responsive to the immunizing antigen ablates the immune response (i.e. the CD4$^{\text{normal}}$ population that remains after depleting the CD4$^{\text{high}}$ T cells does not proliferate when pulsed (Fig. 2B), nor does it proliferate when cultured with the immunizing antigen (Fig. 4)). Limiting dilution analysis to obtain the precursor frequency of antigen-specific T cells in the CD4$^{\text{high}}$ populations both in vitro and in vivo (Figs. 3 and 5) demonstrates a substantial (100-1,000 fold) enrichment of antigen-specific T cells compared to traditional cloning methods in general (Butterfield et al., 1989; Ford and Burger, 1983; Gebel et al., 1983; Kojima et al., 1988; Livingstone and Fathman, 1987), and compared to our previous results in the SWM/DBA2 system in particular (Weyand et al., 1986). In addition, the CD4$^{\text{high}}$ marker can be used to follow the antigen-specific T cell response in vivo (Fig. 5) and allows a method to study T cells which have a suboptimal response to antigen (Fig. 6). The possibility of studying a variety of T cells with different antigen recognition properties, while holding the antigen dose constant, is a novel approach which bypasses some of the obstacles presented by the traditional method of studying a single high affinity T cell in response to a varying antigen dose. Since we have demonstrated upregulation of CD4 by T cells upon first contact with their nominal antigen in several different antigen systems and in several strains of mice, these findings should have broad applicability in immunology. These results open the possibility of using the CD4$^{\text{high}}$ marker to study a variety of antigen-specific autoimmune T cell responses in vitro and in vivo. For example, to date, the study of suboptimal T cell responses has used T cell clones which respond well to their “nominal” antigen, but respond suboptimally to different doses of an altered peptide ligand (Evavold and Allen, 1991; Evavold et al., 1993; Evavold et al.,
The use of the CD4\textsuperscript{high} marker allows us to invert this approach; using a constant dose of an antigen, we can derive T cells which respond suboptimally to that antigen \textit{in vivo}. It is now well established that low proliferative responder T cells can manifest antigen responses to altered peptides by alterations in cytokine production (Evavold and Allen, 1991; Evavold et al., 1993; Evavold et al., 1992). We anticipate that T cells with suboptimal T cell responses might demonstrate an altered cytokine profile compared to T cells that respond with the "usual" high proliferative response to antigen. As another potential application, since the expression of CD4 increases in T cells following their recognition of antigen and the CD4\textsuperscript{high} T cells can be recovered directly from mice, this method can potentially be applied to isolating autoreactive T cells and identifying the autoantigen recognized by the CD4\textsuperscript{high} autoreactive T cells (Sanderson et al., 1995).

That CD4 upregulation by T cells in response to their nominal antigen has not been previously reported, is somewhat surprising. Many of the earlier studies of T cell activation utilized Jurkat cells or T cell hybridomas, and stimulated them with less specific reagents including lectins, ionomycin, and antibodies; in some of these experiments CD4 was reported to be decreased in expression following activation (Cassel et al., 1983; Kelly et al., 1988; Solbach, 1982). In our own lab, we have observed that PMA and ionomycin stimulation can cause apparent CD4 downregulation (unpublished data). The different outcome of CD4 expression following stimulation with specific antigen versus "stronger" or less specific T cell activating agents may reflect a physiologic difference in the mechanism of activation. Studies using T cell clones have also not shown CD4 upregulation. We derived antigen-specific T cell lines and examined the CD4 expression after 10 days culture; the surviving cells were CD4\textsuperscript{high} as expected from Fig. 1 (not shown). We then compared the CD4 levels of an established T cell clone (11.3, derived from DBA/2 mice by immunization with SWM110-121 followed by alternating rest and activation) at 3 and at 10-14 days following activation; the CD4 levels did not change regardless of the activation state (not shown). From these experiments we conclude that T cell clones have already upregulated CD4 expression, which explains why CD4 upregulation was not seen in studies involving T cell clones. These results also suggest that CD4\textsuperscript{high} may be a persistent phenotype marking memory-like cells, which is supported by LDA experiments showing antigen-specific cells in the CD4\textsuperscript{high} group \textit{in vivo} for at least as long as 4 weeks after immunization (M.Fassb, manuscript in preparation).

Although not studied here, it is tempting to speculate upon the biological significance of CD4 upregulation in the T cell response to antigen. The role of CD4 as a coreceptor has been persuasively argued by Janeway, who proposed that the association of CD4 with the TCR during the process of T cell activation could allow enhanced signal amplification (Janeway, 1991; Janeway, 1992). In contrast, naive T cells showed no association of CD4 with the TCR (Dianzani...
et al., 1992). In the CD4 coreceptor model, the increased CD4 expression we have demonstrated could provide a mechanism for enhanced signal transduction via a stoichiometric increase in the amount of CD4 in the TCR activation complex. Another view of the role of CD4 in TCR signalling has been proposed by Germain and coworkers (Konig et al., 1996), in a density model of partial agonist signalling, who suggested that low vs. high densities of CD4 could affect the dissociation time of the TCR with its peptide:MHC complex (Konig et al., 1996). In the density model, the higher CD4 surface expression that we have demonstrated on activated antigen-specific T cells could enhance signalling capability via enhancing overall T cell:MHC/peptide avidity. These and other models of the effect of increased CD4 expression on antigen-specific activated T cells may be amenable to experimentation using the approach described in this paper.

ACKNOWLEDGMENTS

The authors wish to acknowledge Dewey Kim and Dr. Michael Shaw for reading and discussing the manuscript, members of the Herzenberg FACS development team for helpful discussions, Robyn Kizer for her excellent secretarial assistance, and Ms. Cariel Taylor for technical assistance.
Figure 1. Expansion kinetics of $\text{CD}^{4\text{high}}$ T cells during culture.

Lymph nodes cells from DBA/2 mice immunized with SWM(110-121) were cultured with or without Ag (see Materials and Methods) and analyzed periodically by FACS for CD4 and CD44, CD62L, CD69, and CD45RB expression. Dead cells were excluded by PI gating. The CD4$^{\text{high}}$ population is indicated by box outlines, numbers reflect percentages of remaining live cells. The CD4$^{\text{high}}$ population expresses typical activation/memory markers; CD44$^{\text{high}}$, CD69$^{\text{high}}$, CD62L$^{\text{low}}$, and CD45RB$^{\text{low}}$ are shown. One representative experiment of five is shown.
Figure 2. The proliferative response to antigen is contained within the CD4<sub>high</sub> population in culture.

SWM(110-121) primed lymph node cells were cultured as described in Materials and Methods. After 3 days, the response of 500,000 whole lymph node cells was assayed by [3H] thymidine incorporation (A), while a separate aliquot of cells was sterile sorted by flow cytometry into CD4<sub>high</sub> and CD4<sub>normal</sub> populations. 7.5 x 10<sup>4</sup> cells were plated and pulsed immediately with [3H] thymidine for 18 hours before counting on a Beta plate (B). Error bars are the SEM; one representative experiment of five shown.
Figure 3. Limiting dilution analysis quantitates the precursor frequency of CD4^{high} cells.

SWM(110-121) primed lymph node cells were cultured and sorted as described in Figure 2. Titrated numbers of sorted CD4^{high} and CD4^{normal} cells were added to 96 well plates with irradiated spleen and Ag for culture (see Material and Methods). The number of cells/plate which averaged one Ag-reactive cell per well was calculated by Poisson statistics. A total of 1 in 5.1 In this experiment 1 in 5.1 CD4^{high} cells were antigen-specific in this experiment. One representative experiment of five is shown.
Figure 4. **CD4\textsuperscript{high} cells contain the Ag-reactive cells in vivo.**

Freshly harvested SWM(110-121)-immunized DBA/2 lymph node cells were stained and sorted for $1.2 \times 10^4$ CD4\textsuperscript{high} Vβ8\textsuperscript{+} (≤ 0.5% of lymph node cells) or CD4\textsuperscript{normal} Vβ8\textsuperscript{+} cells, which were then cultured for 3 days with irradiated DBA/2 spleen plus SWM(110-121) before pulsing with [\textsuperscript{3}H] thymidine. Error bars represent the SEM; one representative of at least three experiments shown.
Chapter 1  T cells up-regulate CD4 in response to their nominal antigen

Figure 5. LDA of CD4<sup>high</sup> cells allows for the quantification of immune response kinetics in vivo.

CD4<sup>high</sup> Vβ<sup>8+</sup> cells were sorted directly from freshly harvested SWM(110-121)-primed lymph nodes as described in Figure 4 (A). The CD4<sup>+</sup>Vβ8<sup>+</sup> population was 15% of total CD4<sup>+</sup> cells in the presorted lymph node (A). The gates were determined as the brightest 1.3% of all lymph node cells from the CD4<sup>+</sup>Vβ8<sup>+</sup> subset by presort analysis, which represented the brightest 3.7% of CD4 cells from that subset. A shows the FACS plot of the immunized lymph node and data on 5 x 10<sup>3</sup> cells from the sorting subset collected during the sort. The cells were plated and restimulated (see Material and Methods) and the precursor frequency calculated by linear curve fitting according to Poisson statistics (B). One representative experiment of five shown.
Figure 6. Rank ordering of the proliferative response of the progeny of single CD4\textsuperscript{high} T cells obtained by limiting dilution to fixed Ag dose demonstrates heterogeneity of immune response \textit{in vivo}.

After sorting, culturing and counting \(^{3}\text{H}\)thymidine incorporation of CD4\textsuperscript{high} V\(\beta\)8\(^+\) cells sorted directly from SWM(110-121)-immunized DBA/2 mice, an LDA was performed (as in Figs. 3 and 5), and then the proliferative responses of individual positive wells from a plate receiving ~ one Ag-specific cell/well were plotted in rank order. The CD4\textsuperscript{high} Ag-reactive T cells demonstrated equal numbers of cells over a wide distribution (~3 logs cpm) of proliferative response to a fixed Ag dose.

CHAPTER II

Affinity Selection and Loss of Diversity of the TCR Vβ Repertoire during the Course of an Immune Response in Vivo

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Grant Support

This work was sponsored by the National Institutes of Health grants DK39959 and DK44837. William Ridgway was supported by the Howard Hughes Medical Institute.
SUMMARY

We recently described a novel way to isolate the antigen-specific CD4+ T cell population in vivo, following immunization with a nominal antigen, SWM110-121. Phenotypic and functional analyses demonstrated that a diverse antigen-specific SWM 110-121 reactive CD4+ T cell repertoire was activated at the beginning of an immune response, but within 6 days of immunization, a population of oligoclonal, intermediate affinity T cells emerged, which persisted for at least four weeks following immunization and dominated the secondary immune response. These data provide evidence that T cell affinity selection acts early in the immune response in vivo and suggest that persistence, as well as expansion of a population of oligoclonal, intermediate affinity T cells, is involved in CD4+ T cell memory.
INTRODUCTION

The defining characteristic of the adaptive immune system is its ability to mount specific immune responses. T and B lymphocytes mediate the specificity of adaptive immunity via cell surface receptors which recognize specific antigens. Clonal selection, a concept introduced by Burnet to explain the progressive maturation of the lymphocyte repertoire, describes the capacity of individual B cells, expressing unique antibody cell surface receptors, to expand following immunization (Burnet, 1959; Burnet, 1976). The clonal selection theory underlies our current understanding of the ability of the adaptive immune system to mount an accelerated and enhanced immune response following a second encounter with antigen; immunological memory (Ahmed and Gray, 1996; Gray, 1993; Gray, 1994; Gray et al., 1996; Kundig et al., 1996; Mackay, 1993; Sprent and Tough, 1994; Vitetta et al., 1991; Zinkernagel et al., 1996; Zinkernagel et al., 1997). In the B cell compartment, immunologic memory consists of immunoglobulin isotype switching and somatic hypermutation (which leads to an increase in antibody affinity) during the secondary response to antigen, and antigen driven selection of “high affinity” B lymphocytes in germinal centers (Berek et al., 1991; Cumano and Rajewsky, 1986; Jacob and Kelsoe, 1992; Jacob et al., 1991; Jacob et al., 1992; Jacob et al., 1993).

Less is known about clonal selection in the CD4+ T cell compartment. Most evidence suggests that CD4+ T cells do not require the germinal center micro-environment to establish memory (Lane et al., 1994) and do not undergo isotype switching or somatic mutation (Davis and Bjorkman, 1988; Goverman et al., 1986; Hackett et al., 1992; McHeyzer-Williams et al., 1996). The question of whether CD4+ T cells also undergo a process of clonal selection during the course of an immune response in vivo has been difficult to study, because of technical limitations in identifying the very low number of antigen-specific T cells in vivo [1/50,000-300,000 in an unprimed, 1/1000-5000 in primed mice (Ford and Burger, 1983; Gebel et al., 1983; Kojima et al., 1988; Weyand et al., 1986)]. The study of the CD4+ T cell response in vivo has mainly relied upon adoptive transfer of T cell clones or studies using cells from T cell receptor (TCR)-transgenic mice (Garside et al., 1998; Kearney et al., 1994; Khoruts and Jenkins, 1996; Pape et al., 1997). However, all of the transferred T cells shared the same TCR, thus no direct studies could be performed on TCR repertoire development.

Recent work has begun to elucidate the process which leads to the development of the CD4+ T cell repertoire in vivo. The combination of flow cytometry and gene amplification of particular dominant TCR V genes has allowed the examination of the development of an immune response in a non-transgenic animal (Davis et al., 1995; MacDonald et al., 1993; McHeyzer-Williams et al., 1996; McHeyzer-Williams and Davis, 1995; Reiner et al., 1993; Walker et al.,
1995; Walker et al., 1996), and PCR run-off assays identified dominant T cell clonal expansions (Cibotti et al., 1994; Cochet et al., 1992; Dietrich et al., 1994; Musette et al., 1994). However, the question of when and how a dominant, restricted repertoire was selected during the CD4+ T cell immune response remained unanswered.

Recently, we described a novel way to isolate the murine antigen-specific CD4+ T cell subpopulation, both in vitro and in vivo (Ridgway et al., 1998). We used the DBA/2 response to sperm whale myoglobin peptide (SWM) 110-121 to show that, upon antigen challenge, CD4+ T cell up-regulated surface expression of CD4, and that the CD4high subpopulation in vivo, representing less than 1% of the CD4+ population, contained the entire population of antigen-specific T cells. Earlier studies from our lab characterized the CD4+ T cell response to SWM110-121 in DBA/2 mice and have shown utilization of a limited pool of T cell receptor Vß-chains (Ruberti et al., 1991; Ruberti et al., 1991; Ruberti et al., 1993; Ruberti et al., 1992; Sellins et al., 1992). The vast majority (>90%) of CD4+ T cell clones and hybridomas derived from SWM 110-121 immune DBA/2 mice used TCR Vß8.2 with a canonical third complementary determining region (CDR3) consisting of the amino acid sequence (A/G)WDWx(x) linked to the junctional region Jß2.6 (Danska et al., 1990; Sellins et al., 1992). Here we characterize the CD4+ T cell clonal selection process using the CD4high marker to isolate the entire SWM110-121-specific T cell population (not only the dominant responders) at sequential time points in vivo following immunization. Changes in the CD4+ T cell repertoire were studied by isolating CD4high T cells at different time points after primary and secondary immunization and by performing limiting dilution cultures. Our results suggest that an extremely diverse antigen-specific CD4+ T cell response is present at the beginning of the immune response (day 3 after immunization), from which intermediate affinity subsets expand during the course of the immune response. Functional and phenotypic analyses of these subsets suggest that selection is taking place in the CD4+ T cell compartment very early in the immune response, which results in the selection of an intermediate affinity antigen-specific T cell repertoire. The memory CD4+ T cell response reflects an increase in frequency (or persistence) of these intermediate affinity CD4+ T cells, not a selection for the highest affinity CD4+ T cells.

MATERIAL AND METHODS

Animals

DBA/2 mice were purchased from Jackson laboratories (Bar Harbor, ME). Females from 8 to 12 weeks of age were used in all experiments.
Peptide

Sperm whale myoglobin peptide 110-121 (AIHVLHSRHPG) was prepared and HPLC purified at the Protein and Nucleic Acid Facility, Beckman Center, Stanford University.

Antigen proliferation assays

For proliferation assays with T cell clones, \(2.5 \times 10^4\) T cells were incubated in 96 well plates in either T cell media alone or containing two-fold or four-fold dilutions of SWM 110-121 ranging from 0.003 to 150 \(\mu\)M and 5 \(\times\) 10^5 freshly irradiated syngeneic splenocytes. T cell media consisted of RPMI 1640 supplemented with 2 mM L-Glutamine, Penicillin and Streptomycin, nonessential amino acids, sodium pyruvate, and 10 mM hepes buffer (Gibco Laboratories, Grand Island, NY), 5 mM 2-ME (Sigma Chemical Co., St Louis MO) and 5 % FCS. After 48 h at 37°C, 5% CO\(_2\), cells were pulsed with 1 \(\mu\)Ci of [\(^3\)H] thymidine and harvested 18 hours later for counting on a Beta plate (Wallac Inc., Gaithersburg, MT). Cell proliferation, as counts per minute (cpm), was plotted against antigen concentration and ED\(_{50}\) values were derived by calculating the intercept of the antigen concentration leading to half-maximal proliferation. In case of non-canonical T cell clones which did not reach a plateau of proliferation at 150 \(\mu\)M of antigen, the lowest possible calculated ED\(_{50}\) was assigned and thus may be an underestimate (see also Fig. 5). For each T cell clone, a mean ED\(_{50}\) value was calculated from at least two independent proliferation assays. To calculate the statistical significance in ED\(_{50}\) values differences between both canonical and non-canonical T cell populations at day 3 after primary immunization as well as between canonical T cell clones from day 6 after primary and day 4 after secondary immunization, a two sample t-test, assuming unequal variances, was performed using Excel (Microsoft Corporation).

Antibody-inhibition assays

For antibody-inhibition assays with T cell clones, \(2.5 \times 10^4\) T cells were incubated in 96 well plates containing 5 \(\times\) 10^5 freshly irradiated syngeneic splenocytes, 30 \(\mu\)M SWM 110-121 and two-fold dilutions of anti-V\(\beta\)8 antibody (F23.1) ranging from 0 to 2.5 \(\mu\)g/ml. The same media was utilized as in the proliferation assays. After 48 h at 37°C, 5% CO\(_2\), cells were pulsed with 1 \(\mu\)Ci of [\(^3\)H] thymidine and harvested 18 hours later for counting on a Beta plate (Wallac Inc., Gaithersburg, MT). Percentage of inhibition of proliferation was calculated for each dose of anti-V\(\beta\)8 Ab as follows: \([\text{proliferation (cpm) without any added anti-V}\beta\text{8 Ab}]-[\text{proliferation (cpm) with anti-V}\beta\text{8 Ab}]\)/[\text{proliferation (cpm) without any added anti-V}\beta\text{8 Ab}].
Chapter II  TCR affinity selection during an immune response in vivo

FACS and Limiting Dilution Analysis

Groups of 2-3 mice were immunized at the base of the tail with 100 µg SWM 110-121 in complete Freund’s adjuvant (CFA). 3, 6, 8, 14 and 28 days later, draining inguinal lymph node (LN) cells were removed and single cell suspensions prepared. For studying the memory response, DBA/2 mice were first immunized with 100 µg SWM in CFA at the base of the tail, and 4 weeks later, challenged through the same route with 100 µg of the same peptide in incomplete Freund’s adjuvant (IFA). 2, 4, 6, 9 and 14 days later, draining inguinal LN cells were removed and single cell suspensions prepared. The total number of lymph node cells was determined by counting 3 times with a hemocytometer. 4 x 10^7 cells were stained for 20 min at 4°C with 10 µg phycoerythrin (PE)-conjugated anti-CD4 antibody (Caltag, San Francisco, CA) and with 10 µg fluorescein (FITC)-conjugated anti-Vβ8 antibody (F23.1, Pharmingen, San Diego, CA) in FACS buffer (Dulbecco’s PBS with 2% FCS). The cells were analyzed by FACS and the highest 1% of CD4^{high}/Vβ8^{+} or CD4^{high}/Vβ8^{−} subsets were sterile sorted. After sorting, small aliquots of positive and negative populations were resuspended in FACS buffer and analyzed on the FACS machine used for sorting to assess for percent purity of the sorted populations. The cells were placed in limiting dilution culture at 96 replicates per dilution in microwells containing 5 x 10^5 irradiated DBA/2 spleen cells with 10 µM SWM and 10 U/ml IL-2 final concentrations. After 10 days incubation (37°C, 5% CO₂), individual microwells were resuspended and divided equally into two new wells. Freshly irradiated DBA/2 spleen cells (5 x 10^5 /well) were then added either in the presence or absence of 10 µM SWM without IL-2. Cultures were incubated 48 h longer and pulsed with 1 µCi of [³H] thymidine during the last 18h. Positive wells were defined as those exceeding the mean cpm in the absence of antigen by 3 standard deviations (SD). The percent negative wells was plotted against cells per plate and analyzed by least-squares linearization using Cricket Graph (Computer Associates, Inc.). The exponential curve-fitting function produced an equation of the form y=(α)x 10(z)(x). α and z were derived from the data, and the resultant precursor frequency (1/x) was calculated by setting y = 0.37 according to Poisson statistics. The total number of antigen-specific T cells present in the lymph node of each mouse after each immunization (n) was calculated as follow: n=(z/100)/x, where z is the total number of cells present in the immunized lymph node and 1/x is the precursor frequency of SWM110-121-specific T cells in the CD4^{high} population.

LDA T cell clones

At selected time points, duplicate plates were cultured at the time of restimulation; one plate was maintained in culture while the other one was used for proliferative response. LDA T cell clones were subsequently grown from LDA plates which, according to the Poisson statistical
analysis, corresponded to 1 cell per well or less. LDA T cell clones were restimulated every 10-14 days with 20 × 10⁶ freshly irradiated DBA/2 spleen cells and 10 μM SWM final concentration. LDA T cell clones were derived from three independent immunizations.

**TCR Vβ PCR and Sequence analysis**

T cells were Ficoll purified 3 days after restimulation with SWM 110-121 and PolyA⁺-mRNA was prepared using the Microfast Track™ method (Invitrogen Corporation, San Diego, CA). First strand cDNA synthesis was performed on 2 μg of mRNA using an oligo (dT) primer (Promega, Madison, WI) and Moloney Murine Leukemia Virus Reverse Transcriptase (GibcoBRL, Life Technologies, Gaithersburg, MT). 4% of the reaction was used as a template for PCR amplification. The 5' TCR Vβ8 primer was 5'-TAA GCG GCC GCG AGG CTG CAG TCA-3' and the 3' Cβ primer was 5'-CAG CTC AGC TCC ACG TGG TC-3'. The primer annealing was performed at 55°C for 1 min, the polymerase extension segment at 72°C for 2 min, and the junctional region of the Vβ8 containing transcripts was amplified using these two primers in a thermal cycler (Perkin Elmer Corp., Norwalk, CT) for 30 cycles. PCR products were directly sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Life Science Inc., St. Louis, MO) and displayed on a 6% acrylamide gel.

**RESULTS**

**Kinetics of the antigen-specific T cell response to SWM110-121 in vivo**

At various time points after primary or secondary immunization with SWM 110-121, lymph node cells from DBA/2 animals were harvested and stained for CD4 and Vβ8 expression. Limiting dilution analysis (LDA) established the precursor frequency of both CD4<sup>high</sup>/Vβ8<sup>+</sup> and CD4<sup>high</sup>/Vβ8<sup>−</sup> T cell populations at each selected time point. Data presented in Table 1 show the results of one representative LDA experiment performed at each selected time point for the CD4<sup>high</sup>/Vβ8<sup>+</sup> population. The total number of antigen-specific T cells present in the draining lymph node was estimated from the calculated precursor frequencies and absolute numbers of CD4<sup>+</sup> T cells in the node (Table 1). The result of repeated LDA experiments is summarized in Figure 1, which shows the relative expansion of antigen-specific T cells during the course of the primary and secondary immune response in both the CD4<sup>high</sup>/Vβ8<sup>+</sup> and CD4<sup>high</sup>/Vβ8<sup>−</sup> populations. The antigen-specific precursor frequency of the CD4<sup>high</sup>/Vβ8<sup>+</sup> T cells at day 3 after primary immunization (1/3150) was near a baseline level at 33 antigen-specific T cells in the immunized lymph node (Ford and Burger, 1983; Gebel et al., 1983; Kojima et al., 1988; Weyand et al., 1986). In the next
three days, the number of antigen-specific T cells in the CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} population increased rapidly to reach a maximum, at day 6, of over 4,400 CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} T cells in the draining lymph node, corresponding to a 136 fold expansion in the number of antigen-specific CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} T cells from day 3 (Fig. 1). The number of antigen-specific T cells decreased 3 fold by day 8, and by day 28, only 5 % of the peak antigen-specific CD4\textsuperscript{*} T cell number was still present (Fig. 1). The baseline precursor frequency in the CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} population immediately prior to secondary challenge was 6.6 fold higher than the number of antigen-specific T cells present at baseline, day 3 after primary immunization (Fig. 1).

To explore characteristics of the CD4\textsuperscript{*} T cell memory response, mice were re-immunized 28 days after primary immunization and the memory response was studied. The CD4\textsuperscript{*} T cell recall response reached its maximum precursor frequency 4 days after secondary challenge, two days earlier than in the primary response (Fig. 1). The total number of antigen-specific CD4\textsuperscript{*} T cells present at days 4 and 6 of the memory response was not statistically different from that seen at the peak of the primary response, and corresponded to a 24 fold expansion of the existing CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} T cells present 28 days following priming. Not only was the memory response more rapid than the primary response, but it was more sustained after the peak of the response was reached, taking longer for the number of CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} T cell precursors to fall to levels similar to those seen following the primary response (Fig. 1). The CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} population kinetics were similar to the CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} kinetics, although the relative cell expansions were less. There was a 13.5 fold expansion in antigen-specific CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} T cells between day 3 and day 6 of the primary response but, similar to the CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} population, by 4 weeks after primary immunization the precursor frequency of antigen-specific T cells in the CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} population decreased to 5.5 % of the peak cell number (Fig. 1). The memory response in the CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} population was also more rapid and enhanced than the primary response with a 37.5 fold increase in the number of precursors in the first 4 days after re-immunization and a peak which was two and a half times higher than the peak of the primary response (Fig. 1). Data presented in Figure 1 show the dominant expansion of the CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} population compared to the CD4\textsuperscript{high}/V\textbeta8\textsuperscript{*} population. The presence of a minor but significant antigen-specific response in the V\textbeta8\textsuperscript{*} population of sorted CD4\textsuperscript{high} cells was surprising; previously our lab had only rarely been able to isolate SWM110-121-reactive V\textbeta8\textsuperscript{*} CD4\textsuperscript{*} T cells from bulk cultures \textit{in vitro} (Danska et al., 1990; Ruberti et al., 1991; Ruberti et al., 1991; Ruberti et al., 1993; Ruberti et al., 1992; Sellins et al., 1992).
CD4<sup>high</sup> T cells represent a heterogenous population of T cells early in the immune response

The nature of the response of a T cell population to a specific antigen can be estimated by examining the proliferative response of representative individually responding T cells in the population. To test the hypothesis that the proliferative response of the T cell population differed at day 3 and day 6, we produced a representative population of T cell clones by sorting CD4<sup>high</sup>/Vß8<sup>+</sup> T cells directly from day 3 and day 6 lymph nodes, performing LDA and selecting cells for expansion from plates with precursor frequencies approximating one cell/well as calculated by Poisson statistics. These LDA T cell clones were tested for proliferative response. The proliferative responses of the day 3 LDA-derived clones, displayed in rank order, represented over 3 logs of distribution in their ED<sub>50</sub> values (Fig. 2A). The ED<sub>50</sub> values were stable in repeated assays (SEM ≤ 10%). The nature of the rank order distribution of ED<sub>50</sub> values in the day 6 group differed from the day 3 group; most of the responses were near a mean ED<sub>50</sub> of 1 μM with a few outliers on each end (Fig. 2B). This analysis of ED<sub>50</sub> distribution suggested that by day 6 after primary immunization, expansion of intermediate affinity antigen-specific precursors had taken place.

TCR sequence analysis of the antigen-specific TCR repertoire during the primary and secondary response

To ask whether clonal expansion had occurred between day 3 and day 6 of the primary response (as suggested by the emergence of a dominant ED<sub>50</sub> value in the population, Fig. 2), we sequenced the junctional regions of the TCR Vß chains of the Vß8<sup>+</sup> LDA clones. Almost half of the Vß8<sup>+</sup> LDA clones (42.6%) isolated at day 3 displayed non-canonical CDR3 junctional regions (Fig. 3A). However, in agreement with previous studies from our lab, data presented in Figure 3B demonstrate that the majority (87.7%) of LDA clones found at the peak of the primary immune response (day 6) displayed the previously described canonical third complementary determining region (CDR3), consisting of the amino acid sequence (A/G)WDWx(x) linked to the junctional region Jß2.6 (Danska et al., 1990; Sellins et al., 1992) (Fig. 3B). Within the canonical group, 53.1% of the LDA clones displayed the identical specific clonotype AWDW7GS (Fig. 3B). The "memory" T cell clones found 4 weeks after primary immunization displayed a majority (86.4%) of canonical CDR3 regions, however, no preference for a specific clonotype was found (Fig. 3C). In contrast, at the peak of the recall response, not only did the majority (82.7%, 43/52) of the LDA clones examined contain canonical CDR3 regions, but an obvious preference for the specific clonotype Vß8.2-AWDWES-Jß2.6 (59.6%, 31/52) was also found (Fig. 3D).
**Functional analysis of the antigen-specific TCR repertoire during the primary and secondary response**

Since the amino acid composition of the CDR3 loop of most canonical LDA clones isolated at day 6 was similar to the canonical clones seen at day 3 (Fig. 3A-B), we asked whether a functional difference between canonical and non-canonical LDA T cell clones was responsible for the expansion of clones containing the canonical CDR3 region observed between day 3 and day 6 of the primary immune response. Our lab has shown that avidity differences in TCR:Ag:MHC interactions lead to shifts in $ED_{50}$ values (the antigen dose required to reach half-maximal proliferation), with lower avidities leading to higher $ED_{50}$ values and vice versa (Kim et al., 1996). In the present experiments, antigen dose and the affinity of the peptide for the MHC were constants, and the TCR density was comparable on sampled T cell clones by FACS analysis (data not shown). Therefore the system described here could be used to provide information about the affinity of the TCR of the T cell clones for the antigenic ligand SWM 110-121, by measuring their $ED_{50}$ values in a proliferation assay.

Forty-four LDA clones from day 3 cultures and forty-four LDA clones from day 6 cultures, after primary immunization, were tested for proliferation in response to SWM110-121 to determine their $ED_{50}$ values. In general, T cell clones with low $ED_{50}$ values (less than 2 µM) contained the canonical CDR3 region while T cell clones displaying high $ED_{50}$ values (greater than 10 µM) were only seen in LDA clones expressing a non-canonical CDR3 region. A selected panel of LDA clones derived from day 3 cultures is represented by data shown in Figure 4. Three LDA clones which displayed lower $ED_{50}$ values and three clones which displayed higher $ED_{50}$ values are shown, as well as their respective TCR Vβ junctional region sequences.

In order to correlate the $ED_{50}$ value with the affinity of the TCR for the antigenic ligand, it was necessary to demonstrate that the observed $ED_{50}$ values resulted from a TCR signal rather than TCR-unrelated signals (e.g. co-stimulatory molecules). Although comparable expression of major co-stimulatory molecules including CD28, B7-1, B7-2 and CD4, on T cell clones displaying higher and lower $ED_{50}$ values could be seen by FACS analysis (data not shown), a more direct assay was performed to demonstrate the role of the TCR in determining the proliferative capacity of the clones. Titrated doses of anti-Vβ8 antibodies were used in antibody inhibition assays to inhibit the proliferative response of the T cell clones (which all used the Vβ8 TCR). Antibody-inhibition experiments were performed on Vβ8+ T cell clones displaying different $ED_{50}$ values [over a range of two logs (0.07 µM-60 µM)] (Fig. 4A-B). Data presented in Figure 5A show inhibition curves for representative T cell clones, all with different $ED_{50}$ values. At 30 µM SWM 110-121, a lower $ED_{50}$ value of the T cell clone (higher affinity) necessitated a higher antibody dose to reach the
same threshold of inhibition (Fig. 5A). Data presented in Figure 5B show the results of antibody-inhibition assays for all T cell clones tested. A linear inverse correlation was found between the ED$_{50}$ value of a T cell clone and the percent inhibition of the proliferative response. T cell clones displaying low ED$_{50}$ values were inhibited less at a given dose of antibody than T cell clones displaying a higher ED$_{50}$ (Fig. 5B). Furthermore, inhibition curves of T cell clones displaying comparable ED$_{50}$ values were super-imposable in this inhibition assay. These results demonstrated that the distribution in ED$_{50}$ values observed in the LDA T cell clone population resulted from an effect mediated by the TCR. In other words, the measured ED$_{50}$ values were a direct result of the affinity of a particular TCR for its ligand since TCR density, antigen dose, and affinity of peptide for the MHC were kept constant.

In LDA clones from day 3 cultures, the mean ED$_{50}$ value for all T cell clones with the canonical TCR (n=27/44) was 24 fold lower (higher affinity) than in the non-canonical group (P < 0.0036) (Table 2A). In LDA clones from day 6 cultures, the mean ED$_{50}$ value for all LDA clones displaying the canonical TCR (n=40/44) was 9 times lower (higher affinity) than the group displaying the non-canonical TCRs (Table 2B). The demonstrated expansion of the higher affinity T cell clones correlated directly with TCR sequence, strongly suggesting that affinity selection drove the clonal expansion of T cells containing the canonical TCR CDR3 region. After day 6 of the primary response, no further shift in T cell repertoire towards T cells expressing the canonical TCR was observed; the canonical/non-canonical TCR ratio was the same four weeks after primary immunization and at the peak of the memory response (Fig 3A-D).

We next examined the ED$_{50}$ values of LDA clones expressing the canonical TCR, derived 4 days after secondary immunization, to determine whether a further major shift in ED$_{50}$ values occurred in the memory response. Somewhat surprisingly, the mean ED$_{50}$ of sampled (n=9) LDA clones expressing the canonical TCR sequence obtained at the peak of the secondary response was statistically indistinguishable from that seen in the “canonical group” of day 6 LDA clones, obtained after primary immunization [0.86 + 0.36 μM (P=0.96)]. Within the canonical group itself, however, shifts in the representation of particular clonotypes between the primary and secondary response were observed. The predominant clonotype found at the peak of the primary response V$\beta$8.2-AWDWGS-J$\beta$2.6 was different from the clonotype that predominated at the peak of the secondary immune response (day 4 after secondary immunization), V$\beta$8.2-AWDWES-J$\beta$2.6, which was itself under-represented during the primary immune response (Fig. 3B). Another clonotype, V$\beta$8.2-AWDWGJ-J$\beta$2.6, which was represented on almost half of the clones in the canonical group at the beginning of the primary immune response, was considerably diminished at the peak of the primary response, and was not demonstrably present in the secondary response.
These data demonstrate that additional selection took place within the higher affinity canonical group over the course of the primary and secondary immune response.

**DISCUSSION**

The results presented here provide the first direct demonstration of affinity selection in the T cell compartment during an antigen-specific immune response. To generate these data, we made use of our recent finding, that upon antigen challenge, antigen-specific T cells up-regulate cell surface expression of CD4 (Ridgway et al., 1998). By performing limiting dilution analysis on the CD4\textsuperscript{high} population of primed lymph nodes cells, we were able to determine the kinetics of the primary and secondary T cell immune response to SWM110-121 \textit{in vivo}. We examined both the CD4\textsuperscript{high}/Vβ8\textsuperscript{+} and CD4\textsuperscript{high}/Vβ8\textsuperscript{−} populations. As expected from our previous studies, a dominant SWM110-121-specific response was observed in the CD4\textsuperscript{high}/Vβ8\textsuperscript{+} T cell population. Since the CD4\textsuperscript{high} population consists of activated T cells, we used day 3 after primary immunization as our baseline, since that was the first time that SWM110-121-reactive T cells became apparent in the CD4\textsuperscript{high} population. A rapid increase in antigen-specific T cell precursor frequency was seen in the first 6 days of the primary immune response (Fig. 1). In this system, the number of antigen-specific T cells in the CD4\textsuperscript{high}/Vβ8\textsuperscript{+} population decreased by day 8, and within 2 weeks from the day of immunization, only 5% of the peak precursor number was present, however, this left a 6.6 fold higher baseline precursor frequency of CD4\textsuperscript{high}/Vβ8\textsuperscript{+} T cells at day 28 compared to day 3 (Table 1). The secondary response was more rapid (two days earlier) (Fig. 1) and more sustained than the primary response (Fig. 1). After the peak of the secondary response was reached in the CD4\textsuperscript{high}/Vβ8\textsuperscript{+} T cell population, it took four days for the number of precursors to decrease compared to only two days in the primary response (Fig. 1). Similarly, in the CD4\textsuperscript{high}/Vβ8\textsuperscript{−} T cell population, it took six days for the response to decrease in the secondary phase of antigen reactivity, compared to four days after the primary response. The LDA-cloning technique revealed the expected dominant antigen-specific Vβ8\textsuperscript{+} T cell population, but also demonstrated a Vβ8\textsuperscript{−} antigen-specific T cell population reactive to SWM110-121. This was surprising, since our lab (using T cell clones and hybridomas) had only rarely isolated Vβ8\textsuperscript{−} SWM110-121-specific T cells from bulk cultures (Danska et al., 1990; Ruberti et al., 1991; Ruberti et al., 1991; Ruberti et al., 1993; Ruberti et al., 1992; Sellins et al., 1992). Combined with our previous studies, these data suggest that SWM110-121-specific Vβ8\textsuperscript{−} T cells cannot readily expand in bulk culture when Vβ8\textsuperscript{+} SWM110-121-specific T cells (derived from a primary immunization) are present. It has been shown that the degree of heterogeneity in long term T cell lines is less than in freshly obtained lymph node cells, indicating that a selection process occurs \textit{in vitro} (Gammon et al., 1990).
Although the total number of antigen-specific T cells in the CD4^hi/Vß8^- and CD4^hi/Vß8^+ populations in vivo were comparable during the primary and secondary response, the kinetics demonstrate the dominant expansion of the Vß8^+ subset (Fig. 1). The dominance of the Vß8^+ T cells was not due to the fact that Vß8^+ SWM reactive T cells outnumbered the Vß8^- cells at the onset of the immune response, since the number of total antigen-specific precursors (as determined by day 3 LDA) was 2.7 fold higher in the CD4^hi/Vß8^- population than the CD4^hi/Vß8^+ population (Fig. 1). Selection of Vß8^+ SWM reactive T cells occurred rapidly however, and by day 6, 3.7 times fewer Vß8^- than Vß8^+ cells were present. Between day 3 and day 6 of the primary immune response, we observed a 136 fold expansion in the number of antigen-specific T cells in the CD4^hi/Vß8^+ subset compared to only a 13.5 fold expansion in the CD4^hi/Vß8^- T cells subset (Fig. 1). Therefore, our data suggest that in vivo, SWM110-121-specific Vß8^+ T cells have a selective advantage when compared to SWM110-121-specific Vß8^- T cells.

We have presented three lines of evidence that support the idea that clonal maturation of the T cell repertoire occurs early during the immune response in vivo, and reflects a process of affinity selection of the activated T cells. First, as opposed to the continuous distribution in ED_{50} values of LDA T cell clones isolated at day 3, [which reflected the heterogeneous nature of the population of T cells seen after the primary immunization (Fig. 2A)], the majority of the Vß8^+ LDA clones from day 6 displayed similar ED_{50} values, with only a few outliers on each side (Fig. 2B), suggesting that a selection for Vß8^+ LDA clones had taken place in vivo. Second, TCR sequence analysis performed on LDA clones derived from days 3, 6 and 28 of the primary response, and day 4 of the secondary response, indicated a strong bias towards selecting T cells containing the canonical amino acid composition (A/G)WDWx(x) in their CDR3. Third and last, our ED_{50} analysis showed that the skewing towards a “canonical” T cell population correlated with a selection of T cells which displayed lower ED_{50} values (higher affinity), i.e. a functional change in the T cell repertoire correlated precisely with a structural change (Fig. 5). Anti-TCR antibody inhibition experiments demonstrated that the measured ED_{50} values, in this system, directly correlated with TCR affinity of the T cell clones. This result is in complete agreement with recent work from Davis’ group, which showed using surface plasmon resonance (BIACore™), that the main signal event in T cell activation occurs through TCR engagement, and that there exists an extremely good correlation between affinity/dissociation rates of the TCR with the ligand, [peptide:MHC] complex, and the biological (i.e. proliferative) response of the T cell (Alam et al., 1996; Davis et al., 1998; Davis et al., 1997; Lyons et al., 1996; Matsui et al., 1994; Rabinowitz et al., 1996). Although we found comparable expression of major co-stimulatory molecules (CD28, B7-1, B7-2 and CD4) among T cell clones displaying higher and lower ED_{50} values, we did not formally ask whether differential expression of certain adhesion molecules might increase binding and change the
biological response. While adhesion molecules are very important in bringing two cells together for TCR engagement to occur (Croft and Dubey, 1997; Singer, 1992), however, they are not central in the signaling event (Davis et al., 1998; Davis et al., 1997). Therefore, we conclude that in the present series of experiments, the observed T cell proliferative response was a direct reflection of the TCR affinity for its ligand, allowing the ED$_{50}$ values to approximate TCR affinity.

Interestingly, at day 6 after primary immunization, not only had most of the very low affinity T cell clones (very high ED$_{50}$) disappeared (cells that potentially could not compete successfully for growth during the first expansion of antigen-specific precursors, and were thus diluted out by the faster growing cells), but most of the very high affinity T cell clones (very low ED$_{50}$) had also disappeared. The most likely explanation is that the high affinity clones were eliminated by apoptosis due to high avidity interactions between their TCR and the MHC:peptide complex. In vivo injections of high antigen doses have been demonstrated to induce T cell deletion or apoptosis (Critchfield et al., 1994; Critchfield et al., 1995; Lenardo, 1991; Liblau et al., 1994; Moskophidis et al., 1993). This process, termed “propriocidal regulation”, is thought to represent a homeostatic mechanism to prevent a particular clonotype from unrestrained expansion under strong or continuous antigen stimulation (Critchfield et al., 1995; Liblau et al., 1994). In a recent study, Pearson et al. showed that the strength of signal received by the TCR from the peptide/MHC complex determined whether the activated T cell underwent apoptosis or proliferation in vivo (Pearson et al., 1997). Based on this observation and on quantitative studies from our lab on T cell activation (Kim et al., 1996), we postulate that, since the antigen dose and the affinity of the peptide for the MHC was constant in our system, the TCR affinity for the [MHC:peptide] complex rendered high affinity T cells more susceptible to activation induced apoptosis. If this explanation is correct, in a heterogeneous T cell population, those T cells bearing TCRs with a high affinity for the MHC/peptide complex would be eliminated at the beginning (day 6 in this study) of the response.

Our data suggest that a selection for T cells with an intermediate affinity for antigen takes place at the beginning of the primary immune response. That affinity selection occurred in the T cell repertoire during an antigen-specific immune response had been theoretically proposed but never directly demonstrated (De Boer and Perelson, 1994; De Boer and Perelson, 1995; Fishman and Perelson, 1995; Kelsoe, 1996; McHeyzer-Williams et al., 1996; Steele et al., 1993; Tarlinton, 1997). McHeyzer-Williams et al. suggested that a progressive clonal maturation was taking place by demonstrating that the memory response to pigeon cytochrome c (PCC) was more homogeneous than the primary response in terms of CDR3 loop length and amino acid composition. The kinetics of dominant expansion demonstrated by McHeyzer-Williams et al. in the PCC system were similar to the kinetics shown in this report, but their studies did not provide
evidence for affinity selection (McHeyzer-Williams, 1996; McHeyzer-Williams et al., 1996; McHeyzer-Williams and Davis, 1995). In our system, the TCR repertoire at the peak of the memory response was not demonstrably more homogeneous than the TCR repertoire seen at the peak of the primary immune response. The only time a major change was observed in the proportion of "canonical" to "non-canonical" TCRs was at the beginning of the primary response (from day 3 to day 6). For at least four weeks after primary immunization, no changes in the percentage of "canonical" TCR expression was observed, and the same canonical TCR expression was maintained at the peak of the memory response. Furthermore, the ED$_{50}$ values we found for memory-phase derived T cell clones expressing canonical CDR3 regions were indistinguishable from those of canonical LDA clones isolated on day 6 after primary immunization. These data suggest that the more rapid and enhanced memory response was not due to the ability of memory T cells to proliferate more efficiently upon encounter with the antigen (since they displayed ED$_{50}$ values in vitro comparable to primary LDA clones), but to the elevated precursor frequency present at the end of the primary response. Interestingly, although the overall affinity for antigen was not increased during the secondary immune response, a striking switch in the representation of two different canonical CDR3s, differing in only one amino acid (G to E), was observed between the primary and secondary immune response. As determined by ED$_{50}$ analysis, these two sequences belonged to T cell clones that proliferated identically in vitro (data not shown). This shift in phenotype can in part be explained by the skewed peripheral T cell repertoire present in the naive mouse, as one sequence (AWDWGS) was better represented initially, constituting about half of the canonical T cells at day 3 after primary immunization. Why, however, a third CDR3 sequence, which was highly represented at the beginning of the response disappeared entirely, although it had the same apparent affinity for the antigen as the other two TCR sequences, is not clear.

Although our results demonstrate that an affinity selection takes place at the beginning of the T cell immune response, this process is minor compared to that seen in the B cell compartment, and it is not involved in creating the memory response. This is not surprising since the antigen-specific receptor on T cells, unlike the structurally related antigen-specific immunoglobulin on B cells, is dependent on cell-cell contact with its [peptide:MHC] ligand on the APC surface. The TCR:[MHC:peptide] interaction has been shown to be of lower affinity (Alam et al., 1996; Boniface and Davis, 1995; Corr et al., 1994; Matsui et al., 1991; Matsui et al., 1994; Sykulev et al., 1994; Weber et al., 1992). The interaction of the TCR with its ligand is by nature a transient, reversible interaction, and as such, selection of very high affinity TCRs could lengthen the time of contact permitting TCR recognition and signaling. The TCR transient interaction is highly sensitive and specific; very small deviations in the off-rate of the TCR:[MHC:peptide] interaction have a dramatic impact on the T cell response (Alam et al., 1996; Lyons et al., 1996; Valitutti and
Lanzavecchia, 1997; Viola et al., 1997). Previous investigators have demonstrated that T cell activation can be achieved via a small number of [MHC:peptide] complexes serially engaging many TCRs, thus achieving high TCR occupancy (Valitutti et al., 1995; Viola and Lanzavecchia, 1996). We postulate that if the TCR affinity for the [MHC:peptide] complex was too high (or too low), this would lengthen (or shorten) the interaction time, reducing the number of possible engagements necessary to optimally activate the T cell. Further, as mentioned above, too high a TCR affinity could also lead to propriocidal apoptosis of the T cell. Our observation, that selection for a homogeneous population of T cells with intermediate affinity for the MHC:peptide complex occurs between day 3 and day 6 of the primary immune response and that further selection does not occur in the memory response, may reflect affinity selection of a population of T cells whose TCR affinity for the [MHC:peptide] complex involves on and off-rates which are optimal for TCR signaling.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Dewey Kim and Dr. Dirk Brockstedt for reading and discussing the manuscript, Dr. Rob Tibshirani for help with statistical questions, members of the Herzenberg FACS development team for helpful discussions, Mrs. Robyn Kizer for her excellent secretarial assistance and Ms. Cariel Taylor for technical assistance.
Figure 1. Kinetics of antigen-specific precursor frequencies during the primary and secondary response in vivo.

Mean fold expansion of antigen-specific Vß8+ and Vß8- T cells during the primary and memory response to SWM 110-121, starting at day 3 after primary immunization for the baseline precursor frequency. The total number of antigen-specific T cells present at each given time point was determined by LDA as described in M&M. The mean total number of antigen-specific T cells used to determine the expansion of antigen-specific T cells was the result of two or three independent LDA experiments. The expansion was then calculated by dividing the mean total number of antigen-specific T cells present on a given day by the number of antigen-specific precursor cells present in day 3 cultures after primary immunization.
Figure 2. Distribution of ED$_{50}$ values in LDA T cell clones.

CD$_{4}^{hi}$gh/V$\beta$8$^+$ cells sorted from primed lymph nodes 3 days and 6 days after primary immunization were expanded from limiting dilution cultures. For each selected time point, T cell clones were derived from three independent immunizations. A) 44 LDA clones derived from day 3 and B) 44 LDA clones derived from day 6 cultures after primary immunization were tested for proliferative response, and the mean ED$_{50}$ values plotted in rank order. Each LDA clone was tested at least twice (SEM < 10%) in order to determine a mean ED$_{50}$ (as described in M&H).
Figures 3 A-D. TCR Vβ CDR3 sequence analysis of LDA T cell clones.

CD4<sup>high/Vß8</sup> cells sorted from the primed lymph nodes at day 3, 6 and 28 after primary immunization and at day 4 after secondary immunization with SWM 110-121 were expanded from limiting dilution cultures. For each selected time point, T cell clones were derived from three independent immunizations. TCR Vβ CDR3 sequence analysis was performed on A) 47 LDA clones from day 3 B) 49 LDA clones from day 6 C) 22 LDA clones from day 28 after primary immunization and D) 52 LDA clones from day 4 after secondary immunization. Each clone was tested for antigen-specificity in a proliferation assay (Fig. 4 and data not shown). The alignment is based upon common use of the Vß8 segment. Boxes separate germline sequences from N-region additions. Each TCR Dß and Jß assignment is displayed next to the corresponding sequence. The predicted amino acid sequence is displayed below each corresponding sequence. Sequences which contain the CDR3 amino acid composition (A/G)WDWx(x) (in bold) are listed as “canonical” while the other sequences are considered “non-canonical”. The number of clones displaying a particular clonotypic sequence as well as percentages of canonical and non-canonical sequences are listed.

*No assignment to either a Dß1 or a Dß2 germline sequence could be made.
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<td>1/47 (2.1%)</td>
<td>TGT GCC ACC GCC TGG GAC TGG GGG AGC TCG TAT GAC CAG</td>
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57.4%

| 3/47 (6.4%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |

42.6%

| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |
| 1/47 (2.1%)   | TGT GCC ACC CAG TG GAC TGG AC C TAT GCT GAG CAG TTC | TTC TAT GAA CAG SYE Q |

T cell clones TOIß8 CDR3 region

% T cell clones

42.6%
### Chapter II  TCR affinity selection during an immune response in vivo

<table>
<thead>
<tr>
<th>% T cell clones</th>
<th>TCR Vß8 CDR3 region</th>
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<tbody>
<tr>
<td></td>
<td><strong>Vß8.2</strong></td>
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<tr>
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<tr>
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<td>TGT GCC AGC</td>
</tr>
<tr>
<td></td>
<td>CAS A</td>
</tr>
</tbody>
</table>

### Canonical

- 87.7%

### Non-canonical

- 12.2%

#### 1/49 (2.0%)  
- TGT GCC AGC  | GAG TGG GAC A  |
- CAS E      | MDW K  |
- TGT GCC AGC  | GGT CGG GAC A  |
- CAS V      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |

#### 1/49 (2.0%)  
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |

#### 2/49 (4.1%)  
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |

#### 1/49 (2.0%)  
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |

#### 1/49 (2.0%)  
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
- TGT GCC AGC  | GGT GAC A  |
- CAS G      | GTG F  |
### Chapter II  TCR affinity selection during an immune response in vivo

#### Table

<table>
<thead>
<tr>
<th>% T cell clones</th>
<th>TCR Vβ8 CDR3 region</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><strong>Vß8.2</strong></td>
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<tr>
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<tr>
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<tr>
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<td>TGT GCC AGC GCC TGG GAC GAC GGG GGG AGC</td>
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<td>CAS G W DW G SS S Y E Q Y</td>
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<tr>
<td>1/22 (4.5%)</td>
<td>TGT GCC AGC GCC TGG GAC GAC GGG GGG AGC</td>
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<td>CAS G W DW G SS S Y E Q Y</td>
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<td>1/22 (4.5%)</td>
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<td>CAS G W DW G SS S Y E Q Y</td>
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<tr>
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<td>1/22 (4.5%)</td>
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<tr>
<td>1/22 (4.5%)</td>
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</tr>
<tr>
<td></td>
<td>CAS G W DW G SS S Y E Q Y</td>
</tr>
</tbody>
</table>

#### Diagram

- **Canonical**
  - Vβ8.2
    - 2/22 (9.1%)
    - 3/22 (13.6%)
    - 1/22 (4.5%)
  - Dß2
    - 2/22 (9.1%)
    - 3/22 (13.6%)
    - 1/22 (4.5%)

- **Non-canonical**
  - Vβ8.3
    - 1/22 (4.5%)
  - Dß1
    - 1/22 (4.5%)
    - 1/22 (4.5%)

---

*Note: The diagrams depict the TCR Vβ8 CDR3 region for both canonical and non-canonical sequences.*
### Chapter II  TCR affinity selection during an immune response in vivo

<table>
<thead>
<tr>
<th>% T cell clones</th>
<th>TCR Vß8 CDR3 region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>canonical</strong></td>
<td></td>
</tr>
<tr>
<td>31/52 (59.6%)</td>
<td>Vß8.2: GCC ACC</td>
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<tr>
<td></td>
<td>CAS GT GAC</td>
</tr>
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<td>GCC T A</td>
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<td></td>
<td>Dß2: GCC ACC</td>
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<td></td>
<td>CAS GT G AC</td>
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<tr>
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<td>GCC T A</td>
</tr>
<tr>
<td>8/52 (15.4%)</td>
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</tr>
<tr>
<td>2/52 (3.8%)</td>
<td></td>
</tr>
<tr>
<td>1/52 (1.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>non-canonical</strong></td>
<td></td>
</tr>
<tr>
<td>5/52 (9.6%)</td>
<td>Vß8.2: GCC ACC</td>
</tr>
<tr>
<td></td>
<td>CAS GT G AC</td>
</tr>
<tr>
<td></td>
<td>GCC T A</td>
</tr>
<tr>
<td>1/52 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>1/52 (1.9%)</td>
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<tr>
<td>1/52 (1.9%)</td>
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</tr>
</tbody>
</table>
Figure 4. Proliferative profile of canonical and non-canonical LDA T cell clones.

The proliferative profile of representative canonical and non-canonical LDA T cell clones from day 3 after primary immunization. The sequence of each T cell clone is displayed together with the corresponding ED50 value. Each ED50 listed is a mean of two or more experiments (SEM < 10%). In the case of non-canonical T cell clones which did not reach a plateau of proliferation at 150 µM of antigen, the lowest possible calculated ED50 was assigned and thus may be an underestimate.
Figure 5. Direct correlation between TCR function and ED\textsubscript{50} values.

A) Inhibition of proliferation in response to 30 µM SWM 110-121 by titrated doses of anti-Vß8 Ab. Inhibition curves for 4 representative T cell clones are shown. The mean ED\textsubscript{50} value of each respective T cell clone is 3D3 (ED\textsubscript{50} ≥ 50 µM) 3B5b (ED\textsubscript{50} = 15 µM) 3E11 (ED\textsubscript{50} = 0.6 µM) 3E4 (ED\textsubscript{50} = 0.07 µM).

B) Shown is the correlation between the ED\textsubscript{50} values of nine T cell clones and the percentage inhibition of proliferative response to 30 µM SWM 110-121 when 1.25 µg/ml anti-Vß8 Ab was added to the culture. At ED\textsubscript{50} = 1 µM and ED\textsubscript{50} = 50 µM, three different T cell clones displaying identical ED\textsubscript{50} were tested to determine the % inhibition of proliferation. Each T cell clone was tested at least twice. Error bars represent the SEM.
Table 1. Determination by LDA of antigen-specific precursor frequencies in the Vß8+ population.

The total number of antigen-specific precursor cells present in the lymph nodes during the primary and secondary response to SWM110-12 in the Vß8+ population was derived from the precursor frequency of antigen-specific T cells in the CD4high/Vß8+ subset and from the total number of lymph node cells (z), using the equation \( n = (z/100)/x \). CD4high/Vß8+ cells were sorted from primed lymph nodes at days 3, 6, 8, 14 and 28 after primary immunization and days 2, 4, 6, 8 and 14 after secondary immunization. Limiting dilution analysis (LDA) was performed and the precursor frequency \((1/x)\) was derived by Poisson statistics as described in M&M. Shown are the results of one representative LDA experiment performed at each selected time point.
### Table 2

**A) Statistical analysis of ED$_{50}$ values in V$\beta$8$^+$ T cell clones from day 3 after primary immunization.**

27 T cell clones containing the canonical CDR3 region consisting of the amino acid sequence (A/G)WDWx(x) and 17 T cell clones expressing a non-canonical CDR3 region were tested for proliferation in order to determine their ED$_{50}$ value (antigen concentration leading to half-maximal proliferation). Each clone was tested at least twice. A mean ED$_{50}$ value was calculated for each single clone (the SEM were < 10%). The mean ED$_{50}$ values shown in the table represent the mean of all T cell clones in each group. Statistical analysis was performed as described in M&M.

<table>
<thead>
<tr>
<th>Number of clones</th>
<th>Mean ED$_{50} \pm$ Std err</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>canonical</strong></td>
<td>27/44 (61.4%) 0.74 ± 0.15</td>
</tr>
<tr>
<td><strong>non-canonical</strong></td>
<td>17/44 (38.6%) 19.11 ± 5.4 (p&lt;0.0036)</td>
</tr>
</tbody>
</table>

**B) Statistical analysis of ED$_{50}$ values in V$\beta$8$^+$ T cell clones from day 6 after primary immunization.**

40 T cell clones containing the canonical CDR3 region and 4 T cell clones expressing a non-canonical CDR3 region were tested for proliferation in order to determine their ED$_{50}$ values (as described under A).
CHAPTER III

TCR Affinity for Nominal Antigen Correlates with the Development of a Th1 or Th2 Response in Vivo

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Stanford University School of Medicine, Department of Medicine, Division of Immunology and Rheumatology, Stanford, CA 94305

Grant Support

This work was sponsored by the National Institutes of Health grants DK39959 and DK44837. William Ridgway was supported by the Howard Hughes Medical Institute.
SUMMARY

Based on our finding that activated CD4+ T cells express increased levels of cell surface CD4, we developed a T cell cloning system which allowed us to study the affinity selection processes in the CD4 T cell compartment in vivo. We used this system to demonstrate a direct correlation between T cell proliferative responses, their Vß CDR3 sequences, and the development of a Th1 or Th2 response in a population of CD4+ T cells reactive to SWM110-121 in vivo. In addition, we used the (B6xA)F1 response to self and foreign species variants of myoglobin determinant 67-79 to study the role of the pre-existing T cell repertoire in controlling Th-cell differentiation. The current work, in conjunction with our previous results, suggests that that the Th1/Th2-CD4+ T cell selection processes which occur during an immune response in vivo are driven by the overall avidity of the trimolecular TCR:[peptide:MHC] interaction.
INTRODUCTION

Both genetic and environmental factors have been associated with the selective development of a Th1 or Th2 response in vivo. While the influence of the genetic background on Th subset differentiation was discovered several years ago, very little is known about the mechanisms by which the genetic background controls the development of the Th phenotype (Else et al., 1994; Gorham et al., 1996; Guler et al., 1996; Howard, 1986; Howard et al., 1980; Hsieh et al., 1995; Murray, 1998; Murray et al., 1992; Romani et al., 1993). Data presented in this manuscript suggest a possible role for genetically-based pre-selection of the T cell repertoire on the CD4+ T cell Th phenotype differentiation processes. Environmental factors affecting Th phenotype are better understood, and include antigen dose, route of immunization, the type of adjuvant and the type of antigen presenting cell (APC and costimulatory signals) (Bogen et al., 1993; Charlton et al., 1998; Chen et al., 1994; Constant et al., 1995; Finkelman, 1995; Forsthuber et al., 1996; Fox, 1992; Gajewski et al., 1991; Hosken et al., 1995; Kuchroo et al., 1995; Magilavy et al., 1989; Mosmann and Coffman, 1989; Parish, 1972; Shahinian et al., 1993; Stuber and Strober, 1996; Yeung et al., 1998). At the initiation of an immune response, the presence of IL-12 promotes Th1 while IL-4 leads to Th2 polarization of the responding CD4+ T cells (Abbas et al., 1996; Hsieh et al., 1993; Seder et al., 1992; Seder et al., 1993; Seder and Paul, 1994; Seder et al., 1992; Swain et al., 1991).

More recently, work by several laboratories has suggested a role for peptide structure and [peptide:MHC class II] density on APC in influencing the profile of the cytokine response. By varying the dose of antigen, Constant et al. showed that in response to very low doses of soluble antigen, naive T cells bearing a transgenic TCR specific for moth cytochrome c peptide (MCC 81-103) differentiate toward a Th2 phenotype in vitro, while in response to higher doses of the same antigen, they differentiate into Th1 cells (Constant et al., 1995). Using another TCR transgenic model, Hosken et al. found that at low-medium doses of antigen T cells specific for the ovalbumin peptide 323-339 developed to Th1, while at higher doses Th2 development was favored. In the presence of extremely low doses of antigen, however, Th2 development predominated (Hosken et al., 1995). Studies using altered peptide ligands (APL) demonstrated a direct correlation between binding affinity of the peptide for the MHC class II molecule and the cytokine outcome of the T cell response in vivo. Increasing the affinity of the peptide for MHC class II favored Th1 development while decreasing it induced Th2 differentiation (Kumar et al., 1995; Murray et al., 1994; Pfeiffer et al., 1995). Similarly, when higher ligand densities of the same peptide were used to prime mice, Th1 cells were induced, whereas lower ligand densities lead to Th2 induction (Chaturvedi et al., 1996; Pfeiffer et al., 1995; Schountz et al., 1996). What these studies could not
examine, however, was the effect of varying the TCR structure of a population of T cells which responded to the antigen with a range of TCR affinities.

In the first part of this report we used a newly developed system to address the role of the affinity of the TCR for the [peptide:MHC] complex in the development of a Th1 or Th2 response of a whole population of T cells responding to a specific antigen in vivo. Previously, the correlation between TCR affinity for the peptide/MHC complex and the induction of Th1 or Th2 differentiation had been assessed using a fixed (transgenic) TCR. Using the MCC TCR-transgenic model mentioned above, Tao et al. showed that administration of wild type MCC peptide lead to Th1 differentiation of naive T cells in vitro, while at identical ligand density, APLs binding with lower affinity to the transgenic TCR lead to the generation of Th1 and Th2 cells (Tao et al., 1997). A major obstacle in understanding the Th immune response in vivo was that the assessment of a single TCR response in vitro could not shed light on the population response in vivo.

Recently, we described a method to approach the analysis of a whole CD4+ T cell population response to antigen in vivo. We showed that antigen-specific CD4+ T cells, upon antigen challenge, up-regulate surface expression of CD4 both in vitro and in vivo (Ridgway et al., 1998), which allowed us to follow the development of a T cell immune response in vivo (M. Fassò, submitted). In this system the [antigen:MHC] complex was constant in affinity, structure and dose. We were able to isolate and expand the entire antigen-specific population present in the lymph node at an early stage (3 days after immunization) of the DBA/2 response to sperm whale myoglobin peptide (SWM)110-121, and show that it was considerably more diverse in its response than the predominant response we described in vitro previously. The DBA/2 response to SWM110-121 as previously characterized by our lab was predominantly mediated by T cells expressing the TCR Vß8.2 linked to the junctional region Jß2.6 with the “canonical” amino acid sequence (A/G)WDWx(x) in the third complementary-determining region (CDR3) (Danska et al., 1990; Ruberti et al., 1991; Ruberti et al., 1991; Ruberti et al., 1993; Ruberti et al., 1992; Sellins et al., 1992). At day 3 after immunization, however, we isolated and expanded a panel of Vß8+ T cell clones which expressed “non-canonical CDR3 regions”, and displayed higher ED₅₀ values than Vß8+ T cell clones (derived on the same day) expressing “canonical” CDR3 regions (M. Fassò, submitted). In the present study, we found a direct correlation between the ED₅₀ value of a T cell clone -previously demonstrated to be a reflection of TCR affinity (M. Fassò, submitted)-, its CDR3 junctional sequence, and the selective development towards a Th1 or Th2-like phenotype, shedding new light on the effect of the TCR affinity on the development of Th phenotype during an immune response in vivo.
As a second aspect of the population response to a T cell antigen, we next investigated the role of the overall affinity of the pre-existing T cell repertoire on the development of Th1 and Th2 responses in vivo. Previously, our lab had characterized the (B6xA) F1 immune response to the SWM peptide amino acids 67-79, restricted by I-E^k (Kim et al., 1996). A single substitution at amino acid residue 74 distinguishes the self-determinant, mouse myoglobin (MM) from the foreign variants, sperm whale (SWM) and horse myoglobin (EqM). These three peptides have a similar affinity for the I-E^k MHC class II molecule (Kim et al., 1996). Therefore, administration of identical doses of antigen leads to similar ligand densities on APC for all three peptides. The (B6xA)F1 response to species variants of myoglobin peptide 67-79 provided us with a system where all environmental factors including [antigen:MHC] density were kept constant and where the only variable affecting the commitment to Th phenotype consisted in the selective activation of a self- or foreign-reactive peripheral T cell repertoire. The observation of a dichotomy in Th response against the self- versus the two foreign myoglobin peptides supported the conclusion that TCR affinity in the pre-existing T cell repertoire can determine the cytokine outcome of an immune response in vivo.

MATERIAL AND METHODS

Animals.

DBA/2 and (B6xA)F1 mice were obtained from the Jackson laboratory (Bar Harbor, ME). Females from 8 to 12 weeks of age were used for the experiments.

Peptide.

Sperm Whale Myoglobin peptides 110-121 (AIIHVLRSHRHPG) and 67-69 (TVLTALGAILKKK), Horse Myoglobin 67-79 (TVLTALGGILKKK) and Mouse Myoglobin peptide 67-79 (TVLTALGTILKKK) were prepared and HPLC purified at the Protein and Nucleic Acid Facility, Beckman Center, Stanford University.

Antigen proliferation assay

For proliferation assays with primed lymph node cells, single mice were immunized at the base of the tail with IFA plus 10 mg/ml heat-killed Mycobacterium tuberculosis, H37RA (Difco Laboratories Inc., Detroit, MI) emulsified with an equal volume of 100 μg peptide in CFA. 6-8 days after immunization, draining inguinal lymph node (LN) cells were removed and single cell suspensions were prepared. 5 x 10^5 cells were incubated in 96-well flat-bottom plates in either T cell media alone or with titrated doses of antigen or purified-protein derivative (PPD). T cell
media consisted of RPMI 1640 supplemented with 2 mM L-glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, 10 mM hepes buffer (Gibco Laboratories, Grand Island, NY), 10 mM 2-ME (Sigma Chemical Co. St. Louis MO), and 0.5% normal mouse serum. After 72 h at 37°C, 6% CO₂, cells were pulsed with 1 μCi of [³H] thymidine and harvested 18 h later for counting on a Beta plate (Wallac, Inc., Gaithersburg, MT).

For proliferation assays with T cell lines or clones, 2.5 x 10⁴ T cells were incubated in 96 well plates in either T cell media (complemented with 5% FCS) alone or containing two to four-fold dilutions of antigen from 0.003 to 100-150 μM. After 48 h at 37°C, 5% CO₂, cells were pulsed with 1 μCi of [³H] thymidine and harvested 18 h later for counting on a Beta plate. Cell proliferation, as counts per minute (cpm), was plotted against antigen concentration, and ED₅₀ values were derived by calculating the intercept of the antigen concentration leading to half-maximal proliferation. For each T cell clone, a mean ED₅₀ value was calculated from at least two independent proliferation assays.

Lymphokine secretion assay for Hybridomas

T cell hybridoma cells (5 x 10⁴) and irradiated spleen cells (0.5 x 10⁶) were incubated in 96 well plates in either T cell media (complemented with 5% FCS) alone or containing 10 μM Ag. After 24 h at 37°C, 5% CO₂, the supernatant was harvested, frozen and thawed for assaying IL-2 production using the IL-2 responsive murine T lymphocyte line HT-2. 1 x 10⁴ HT-2 cells were added to the supernatant. The plates were incubated for 20 h at 37°C, 5% CO₂, and then were pulsed with 1 μCi of [³H] thymidine and harvested 18 h later for counting on a Beta plate.

T cell lines and LDA clones

In order to establish T cell lines, single cell suspensions obtained from primed LN were cultured for 4 days in 6-well flat-bottom plates at 2.5 x 10⁶ cells/ml. Live cells were purified on a Lympholyte™ (Accurate Chem., Westbury, NY) gradient, stained with phycoerythrin (PE)-conjugated anti-CD4 antibody (Caltag, San Francisco, CA) in FACS buffer (Dulbecco’s PBS with 2% FCS) and analysed by FACS to determine appropriate sorting gates as described previously (Ridgway et al., 1998). CD⁴⁺ cells were sterile sorted and incubated for an additional 4 days in T cell media with 5 U/ml IL-2 (37°C, 5% CO₂). The T cell lines were restimulated in 6 well plates every 10-14 days with 25 x 10⁶ freshly irradiated syngeneic spleen cells plus 10 μM of the same antigen which was used for the initial priming.

After the third restimulation of (B6xA)F1 T cell lines named “Eq”, “Sp” and “Mu#2”, T cell hybridomas were generated using a protocol previously described (Rock, 1985). Briefly, T cell lines were fused to BW5347αβ with 40% polyethylene glycol and aliquoted in 96-well flat-
Chapter III  TCR affinity correlates with Th1 or Th2 development in vivo

bottom microtiter plates. Hybridomas were selected in hypoxanthin-aminopterin-thymidine medium for 3 weeks and then grown in hypoxanthin-thymidine medium (Life Technologies, Inc., Gaithersburg, MD) for two passages before the change to normal medium.

The DBA/2 T cell clones specific for SWM 110-121 were derived by immunizing groups of 2-3 mice at the base of the tail with 100 µg SWM 110-121 in CFA. 3 and 6 days later, draining inguinal lymph node (LN) cells were removed and single cell suspensions prepared. 4 x 10⁷ cells were stained for 20 min at 4 °C with 10 µg (PE)-conjugated anti-CD4 antibody (Caltag, San Francisco, CA) and with 10 µg fluorescein (FITC)-conjugated anti Vβ8 antibody (F23.1, Pharmingen, San Diego, CA) in FACS buffer (Dulbecco’s PBS with 2% FCS). The cells were analyzed by FACS and the highest 1% of CD4⁺/Vβ8⁺ subset were sterile sorted. The cells were placed in limiting dilution culture at 96 replicates per dilution in microwells containing 5 x 10⁵ irradiated DBA/2 spleen cells with 10 µM SWM and 10 U/ml IL-2 final concentrations. T cell clones were grown from limiting dilution plates which, according to Poisson statistical analysis, corresponded to 1 cell per well or less. T cell clones were expanded by restimulation with 20 x 10⁶ freshly irradiated DBA/2 spleen cells and 10 µM SWM 110-121 final concentration every 10-14 days (37°C, 5% CO₂).

Intracellular Cytokine Staining (ICC)

10-14 days after the last restimulation, T cell lines and clones were purified on a Lympholyte™ gradient (Accurate Chem., Westbury, NY) and activated with 1 µg/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St Louis MO) and 0.25 µg/ml Ionomycin (Sigma Chemical Co., St Louis MO) for 6 hours (37°C, 5% CO₂). After the first 4 hours, 10 µg/ml Brefeldin A (Epicentre Technologies, Madison, WI) was added to the culture. At the end of the incubation cells were then stained for 20 min at 4°C with a predetermined optimal concentration of TRI-COLOR® (R-PE-Cy5 Tandem) (TC)-conjugated anti-CD4 antibody (Caltag, San Francisco, CA) in FACS buffer. The cells were then fixed for 20 min at 4°C with 2% paraformaldehyde in PBS (Sigma Chemical Co., St Louis MO) and then permeabilized for 10 min at room temperature with 0.5% Saponin in PBS (Sigma Chemical Co., St, Louis, MO). Cells were stained in permeabilization buffer (0.5% Saponin in PBS) for 20 min at 4°C with predetermined optimal concentrations of phycoerythrin (PE)-conjugated anti-IFN-γ antibody and either fluorescein (FITC)-conjugated anti IL-4 or fluorescein (FITC)-conjugated anti IL-10 antibodies (Pharmingen, San Diego, CA). As a negative staining control, cells were stained with an isotype-matched control antibody of irrelevant specificity (PE-conjugated rat IgG₁, and FITC-conjugated rat IgG₂, Pharmingen, San Diego, CA) at the same concentration for the anti-cytokine antibody. Cells were washed twice in permeabilization buffer and once in FACS buffer and then analyzed by three-
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color flow cytometry on a Becton Dickinson FACScan cytometer (Mountain View, CA). The Data were analyzed using the Herzenberg desk facility plus Flojo (Tree Star, San Carlos, CA) on a Power Macintosh (Apple Computer, Cupertino, CA).

**TCR Vß PCR and Sequence analysis.**

T cells were purified on a Lympholyte™ gradient (Accurate Chem., Westbury, NY) 3 days after restimulation with SWM 110-121 while T cell hybridomas were simply washed in PBS and PolyA+ mRNA was prepared using the MicroFast TrackTM method (Invitrogen Corporation, San Diego, CA). First strand cDNA synthesis was performed on 2 μg of mRNA using an oligo (dT) primer (Promega, Madison, WI) and Moloney Murine Leukemia Virus Reverse Transcriptase (GibcoBRL, Life Technologies, Gaithersburg, MT). 4% of the reaction was used as a template for PCR amplification. For amplification of the TCR Vß chain of SWM 110-121-specific T cell clones, the 5' TCR Vß8 primer 5'-TAA GCG GCC GCG AGG CTG CAG TCA-3' and the 3' Cß primer 5'-CAG CTC AGC TCC ACG TGG TC-3' were used. To screen and amplify the TCR Vß chain of MM-, EqM-, and SWM 67-79-specific T cell hybridomas, 5' primers specific for Vß1 to Vß19 were used in conjunction to the 3' Cß primer described above. The sequences were Vß1 5'-ATC TAA TCC TGG GAA GAG CAA AT-3'; Vß2 5'-GGC GTC TGG TAC CAC GTG GTC AA-3'; Vß3 5'-GTG AAA GGG CAA GGA CAA AAA GC-3'; Vß4 5'-GAT ATG CAA AGC ATG GTT TAG CTA CAA TAA TA-3'; Vß5 5'-ACA TAA TCA AAG GAA AGG GAG AA-3'; Vß6 5'-TCC TGA TTG GTC AGG AAG GGC AA-3'; Vß7 TAC CTG ATC AAA AGA ATG GGA GA-3'; Vß9 5'-AGC TTG CAA GAG TTG GAA AAC CA-3'; Vß10 5'-GAT TAT GTT TAG CTA CAA TAA TA-3'; Vß11 5'-ACA AGG TGA CAG GGA AGG GAC AA-3'; Vß12 5'-ACC TAC AGA ACC CAA GGA CTC AG-3'; Vß13 CAG TTG CCC TCG GAT CAA TTT TC-3'; Vß14 5'-GCC GAG ATC AAG GCT GTG GGC AG-3'; Vß15 5'-AGA ACC ATC TGT AAG AGT GGA AC-3'; Vß16 5'-CAT CAA ATA ATA GAT ATG GGG CA-3'; Vß17 5'-GTA GTC CTG AAA AAG GGC ACA CT-3'; Vß18 5'-CAT CTG TCA AAG TGG CAC TAC TT-3' and Vß19 5'-AGA CAT CTG GTC AAA GGA AAA G-3'. The primer annealing was performed at 55°C for 1 min, the polymerase extension segment at 72°C for 2 min, and the junctional region of the Vß8-containing transcripts was amplified using these two primers in a thermal cycler (Perkin Elmer Corp., Norwalk, CT) for 30 cycles. PCR products were directly sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Life Science, Inc., St. Louis, MO) and displayed on a 6% acrylamide gel.
RESULTS

TCR Affinity for the [SWM:MHC] complex directly correlates with the polarization to Th1 or Th2 cytokines production in DBA/2 mice in vivo.

As shown in our previous work, it was possible to identify antigen-specific T cells from an animal in vivo based on cell surface CD4 expression levels (Ridgway et al., 1998). 3 days and 6 days after immunization with SWM110-121, we sorted CD4^{hi}/V{\beta}8{\dagger} T cells from the draining lymph nodes (LN) and cultured them directly under limiting dilution conditions. Plating the SWM110-121-specific T cells directly into microwells at less than 1 cell/well right allowed cells which recognized antigen weakly to grow without competing for growth with the better growing/higher affinity T cells. We previously demonstrated that the differential growth observed in T cell clones from day 3 and day 6 cultures, as defined by their ED_{50} values, was a stable phenotype resulting from differences in affinity of the TCR for the antigenic ligand (M. Fasso, submitted).

We selected 10 T cell clones, 4 of which displayed low ED_{50} values (< 1 \mu M) and 6 of which displayed high ED_{50} values (> 10 \mu M) to test for cytokine production (Fig. 1A). Supporting our previous findings, the differences in proliferative response correlated to structural diversity in the TCR, since all the high affinity T cell clones expressed the canonical amino acid sequence (A/G)WDWx(x) in their third complementary-determining region (CDR3) of the TCR V{\beta}8 chain, while all the low affinity T cell clones expressed different, non-canonical amino acid sequences (Fig. 1B) (M. Fasso, submitted). Expression of IFN-\gamma, IL-4 and IL-10 was assayed in each of the T cell clones by intracellular cytokine staining. Data presented in Figure 1A clearly demonstrates that high affinity (low ED_{50} values) T cell clones exclusively produced IFN-\gamma, characteristic of Th1 responses while low affinity (high ED_{50} values) T cell clones mounted Th2 responses with the production of both IL-4 and IL-10 but not IFN-\gamma (Fig. 1A). These data represent the first demonstration of a direct correlation between the affinity of the TCR for the peptide:MHC complex and the development of a Th1 or Th2 response in vivo.


To study the importance of the pre-existing peripheral T cell repertoire in determining the cytokine outcome of a T cell immune response, we immunized (B6xA) F1 mice with self and foreign variants of myoglobin determinant 67-79. A single substitution at amino acid residue 74 distinguishes the self-determinant, mouse myoglobin (MM) from the foreign variants, sperm whale (SWM) and horse myoglobin (EqM) (Fig. 2). The mouse analog has glycine at this position.
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(74G), while the horse and sperm whale have alanine (74A) and threonine (74T), respectively. We generated T cell lines specific for each (foreign and self) determinant. Figure 3 shows a representative dose response curve for each SWM-, EqM- and MM 67-79-specific T cell line. The proliferative response (in terms of antigen dose needed to reach half-maximal proliferation as well as the maximal level of proliferation itself) of the T cell lines raised against the MM 67-79 determinant for their natural peptide was consistently lower than the response of the clones raised against the two foreign determinants SWM and EqM 67-79 (Fig. 3).

In order to distinguish whether the diminished proliferation observed in response to the self-determinant MM 67-79 was the result of low affinity recognition of the self-determinant by the same T cell repertoire that recognized EqM 67-79 and/or SWM 67-79 versus a distinct T cell repertoire, we analyzed the TCR Vβ usage and fine sequence structure of a set of T cell hybridomas derived from T cell lines of each specificity. Both the TCR Vβ and, in most cases, the CDR3 amino acid sequence of the T cell hybridomas were determined, and their reactivity for all three species variants of myoglobin was assayed (Fig. 4A-C). An apparent preference for Vβ6 and Vβ15 was observed in T cell hybridomas specific for SWM-67-79 (90%) and EqM 67-79 (66.6%), although the same Vβs could also be found in almost half of the MM 67-79-specific T cell hybridomas (43.8%). That the self-specific T cell response truly derived from a distinct T cell repertoire was ultimately suggested by sequence analysis of the Vβ CDR3 regions, which demonstrated an entirely non-overlapping set of sequences between the three specificity groups (Figure 4A-C). In addition, a striking difference was observed in the recognition of species variants of myoglobin between the foreign-reactive and self-reactive T cell hybridomas. While only a simple methyl or hydroxyl group distinguished the two foreign variants from the self variant of myoglobin, both SWM- and EqM 67-79-specific T cell hybridomas cross-reacted to SWM and EqM antigens with equal strength. On the other hand, only 50 to 60 percent of SWM or EqM specific T cell hybridomas cross-reacted weakly to MM 67-79. The opposite was true for MM-specific T cell hybridomas: only 31.3 percent of all the MM 67-79-specific T cell hybridomas cross-reacted to both SWM and EqM, and to a minor extent (Fig. 4A-C). The TCR sequence and cross-reactivity patterns strongly suggested that an unique T cell repertoire was expanded after immunization with the MM 67-79 peptide.

We next asked whether peripheral activation by identical ligand densities of self- or foreign myoglobin peptides would differentially affect self- and foreign reactive Th phenotypes in response to priming with self or foreign analogues of myoglobin 67-79. The Th phenotypes of the cell lines were determined by intracellular cytokine staining for the production of IFN-γ, IL-4 and IL-10. Figure 5 shows representative intracellular cytokine staining patterns for individual SWM, EqM and MM 67-79-specific T cell lines. While 3/3 MM67-79-reactive T cell lines displayed a
predominant Th2 pattern of cytokines (production of L-4 and IL-10), FACS analysis revealed a predominant Th1 response (IFN-γ) in both foreign T cell lines (Fig. 4). Therefore, under identical activation conditions (same immunization route, same adjuvants, same ligand densities), which are known to favor Th1 immunity (Forsthuber et al., 1996; Mosmann and Coffman, 1989), only the high affinity foreign-reactive T cell repertoire lead to predominant Th1 responses. The lower affinity, self-reactive T cell repertoire, responded to the presented self-peptide in a predominant Th2 fashion.

**DISCUSSION**

The present work demonstrates a direct correlation between TCR affinity and the development of a Th1 or Th2 response *in vivo*. Data presented here suggests that the development of a Th1 or Th2 immune response *in vivo* depends on the avidity of the TCR:[peptide:MHC] interaction, which directly determines the functional development of the T cell response if all other variables are held constant. Furthermore, the avidity of the TCR:[peptide:MHC] interaction can be limited by the overall avidity of the pre-existing T cell repertoire. We will discuss the significance of these findings in the light of T cell affinity and repertoire selection.

It has been difficult to draw conclusions about the relationship between TCR affinity, ligand structure, and T cell cytokine response. Although studies performed with APLs *in vivo* showed that immunization with APLs, which varied (compared to the wild type peptide) in their affinity for the MHC or for the TCR of cloned T cells, affected the functional Th1 or Th2 outcome of the immune response *in vivo* (Murray et al., 1994; Pfeiffer et al., 1995), this could not be correlated to variations in the overall avidity of the TCR:[peptide:MHC] interaction. Since *in vivo* an entire T cell repertoire of T cells can potentially recognize the antigen, by altering the peptide structure or density, a completely different set of T cells from the repertoire could be selected with “optimal” affinity for the [APL:MHC] complex. Therefore, no direct conclusion could be drawn about the overall avidity of the trimeric complex leading to one or the other Th phenotype *in vivo*. In order to address the role of an avidity mechanism in the Th1 or Th2 selection *in vivo*, two variables of the trimeric TCR:[peptide:MHC] interaction must remain constant and only one should vary. We were able to hold antigen dose (APC surface antigen density) and [MHC:peptide] affinity constant in the present study, while the TCR affinity (a third variable) varied as selected in the pre-existing T cell repertoire.

We were able to explicitly demonstrate the role of the avidity of the TCR:[antigen:MHC] interaction in the differentiation into Th1 and Th2 T cell effector subsets *in vivo* using the CD4^{high}
system, which allowed an inverse approach from previous investigations by maintaining the ligand structure and density constant (i.e. SWM dose and MHC expression were constants) and only effectively varying the T cell affinity for the [peptide:MHC] complex. Experiments with in vivo-derived T cell clones with a range of ED₅₀ values from 0 µM to > 60 µM demonstrated that all high affinity (ED₅₀ ≤ 2 µM), "canonical" T cell clones developed into Th1 responders while all low affinity (ED₅₀ ≥ 10 µM), "non-canonical" T cell clones developed into Th2 responders. In other words, changes in the strength of the avidity interaction between the TCR and their ligand, caused by differences in TCR affinity, directly correlated with the development of one or the other Th-cell effector subset.

According to this avidity model, one also expects that a change in the ligand density (by varying the immunizing dose of antigen) would also lead to a corresponding change in the Th phenotype of the immune response. A number of studies have shown in fact that changes in the immunizing dose of antigen can lead to changes in the phenotype of the immune response (Bretscher et al., 1992; Murray et al., 1994; Murray et al., 1995; Murray et al., 1992; Parish, 1972; Pfeiffer et al., 1991; Pfeiffer et al., 1995; Secrist et al., 1995). As mentioned above, direct evidence that the same set of TCR was elicited when decreasing or increasing the dose of immunizing peptide could not be previously shown. Data presented here suggest that the determining factor in the shift to Th2 phenotype when decreasing the immunizing dose of peptide is not only the amount of antigen but also the overall affinity of the selected T cell repertoire. Our explanation for the observed Th1/Th2 shift is that in the event of very low immunizing doses of antigen, there might not be any T cells with high enough TCR affinity to reach the optimal avidity interaction required for Th1 development, therefore Th2 development will ensue.

The determining role of the T cell repertoire in Th development was directly demonstrated in this work by studying the (B6xA)F1 response to species variants of myoglobin. TCR Vβ sequence analysis as well as analysis of fine specificity demonstrated that distinct T cell repertoires are involved in (B6xA)F1 mice in the recognition of self versus foreign variants of myoglobin 67-79. Since identical concentrations of the three species variants of myoglobin 67-79 have been shown by our lab to lead to similar ligand densities (Kim 1996) the diminished proliferative response observed in vitro in response to immunization with MM67-79 compared to immunization SWM-and EqM 67-79 suggests that the MM67-79-reactive T cell repertoire has a lower affinity for MM 67-79 than do the foreign T cell repertoires for the corresponding foreign peptides. Since we did not provide evidence that the mouse determinant of myoglobin amino acids 67-79 is actually naturally processed and presented on endogenous APCs, nor that MM67-79 acts as a selecting ligand in the thymus, the low affinity response observed upon immunization with self myoglobin might result from two effects. One explanation would be that a "hole" in the repertoire
exists in (B6xA)F1 mice for the recognition of MM 67-79, because of lack of positive selection in
the thymus for that particular repertoire (Schaeffer et al., 1989). If, however, MM 67-79 was
actually processed and presented on thymic APCs, thymic education might have negatively selected
T cells that could engage MM 67-79 with high affinity (Alam et al., 1996; Ashton-Rickardt and
Tonegawa, 1994). Since thymic positive selection occurs upon low avidity recognition of self,
during a peripheral response to a self-myoglobin, a number of cells could still potentially engage
the self-antigen with low avidity. Regardless of which of the two possibilities produced the
particular T cell repertoire response to MM67-79, our data demonstrates that as a result of the low
avidity recognition between the TCR and the [peptide:MHC] complex, the response to MM67-79
in the (B6xA)F1 mice developed into a Th2 phenotype. This Th2 response was not due to a too
low density of presented ligand on APC, since the same ligand density of two foreign determinants
of myoglobin, EqM67-79 and SWM67-79 was able, like SWM110-121 in DBA/2, to lead to a
predominant Th1 response in the same mouse. We conclude that T cells, which at the given ligand
density could have potentially engaged self-myoglobin with such an avidity to promote a Th1
response must have not been present in (B6xA)F1 mice, due to negative, or lack of positive thymic
selection.

The results in both the (B6xA)F1 and DBA/2 immunization models highlight a central role
for thymic selection in determining the development of Th1 or Th2 responses. By determining the
range of affinities of the selected peripheral T cell repertoire for any specific antigen, thymic
selection can control the overall avidity of certain TCR:[peptide:MHC] interactions, resulting in a
predisposition to the development of either a Th1 or a Th2 response following immunization with
antigen. We propose that the overall affinity of the pre-existing TCR repertoire (selected in the
thymus) will be a major genetic determinant in dictating Th1 or Th2 immunity to antigens. Of
course, this phenomenon can be modulated by the previously discussed environmental factors,
e.g. the effect of cytokines present at the initiation of the immune response, and which can
dramatically vary by for example using different adjuvants (Bogen et al., 1993; Charlton et al., 1998;
Forsthuber et al., 1996; Fox, 1992; Mosmann and Coffman, 1989; Yeung et al., 1998) Further
studies will be required to define the hierarchical influence of each particular genetic and
environmental factors in the outcome of the immune response.
Figures 1 A and B. TCR affinity directly correlates with Th1 or Th2-cell subset development.

A) Intracellular cytokine staining of high and low affinity LDA T cell clones specific for SWM 110-121.

The DBA/2 T cell clones specific for SWM110-121 were derived by sorting the CD4^{high} population of draining lymphnodes 3 or 6 days after immunization with SWM 110-121 in CFA and by directly culturing the CD4^{high} population under limiting dilution conditions (as described in M&M). The proliferative profile of all 10 T cell clones is shown. Four LDA T cell clones displaying ED_{50} values < 2 \mu M (high affinity, open symbols) and six LDA T cell clones displaying ED_{50} values > 10 \mu M (low affinity, solid symbols) were tested for cytokine production by intracellular staining (as described in M&M). The names of the T cell clones appear on top of each FACS panel together with the corresponding symbol used in the proliferation assay. IFN-\gamma staining appears on the y axis and IL-4 staining appears on the x axis of each FACS panel. Percentages of cells contained within gated populations are displayed inside each gate. The numbers appearing in parenthesis refer to percentages of cells contained in the same gates, when IL-10 instead of IL-4 staining was displayed on the x axis. Isotype control stainings revealed < 1% cells in both FITC and PE channels.

B) TCR V\beta CDR3 sequence analysis and ED_{50} values of high affinity and low affinity LDA T cell clones specific for SWM 110-121.

TCR V\beta CDR3 sequence analysis was performed on all 10 LDA T cell clones from Figure 1A. The alignment is based upon common use of the V\beta8 segment. Boxes separate germline sequences from N-region additions. Each TCR D\beta and J\beta assignment and mean ED_{50} value is displayed next to the corresponding sequence. The predicted amino acid sequence is displayed below each corresponding sequence. Sequences which contain the CDR3 amino acid composition (A/G)WDWx(x) (in bold) are listed as “canonical” while the other sequences are considered “non-canonical”. The mean ED_{50} values shown in the table represent the mean of at least two independent proliferation assays performed on each particular T cell clone (the SEM were < 10%).
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**Canonical**

**Non-canonical**

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Mouse Myoglobin  
Horse Myoglobin  
Sperm Whale Myoglobin

Figure 2. Amino acid comparison between species variants of myoglobin determinant 67-79.

Amino acid sequence of the myoglobin determinant 67-79 in the horse, sperm whale and mouse.

Dashes represent identical amino acids to the mouse sequence.

Figure 3. Proliferative profile of T cell lines specific to species variants of myoglobin determinant 67-79.

T cell lines were derived by immunization of (B6xA)F1 mice with species variants of myoglobin determinant 67-79 in CFA. Shown is the representative proliferative profile of three independent MM 67-79-specific T cell lines, Mu#1 (solid squares), Mu#2 (solid circles) and Mu#3 (solid triangles), one SWM 67-79 (open squares) and one EqM 67-79 (open circles) -specific T cell lines (Sp and Eq, respectively). The proliferative pattern of the T cell lines was tested after the 4th restimulation and did not vary over the entire time in culture (up to 9 subsequent restimulations).
Table 4. T cell repertoire analysis of T cell hybridomas derived from SMW-, EqM- and MM 67-79-specific T cell lines.

TCR Vβ CDR3 amino acid sequence analysis and pattern of reactivity was performed on T cell hybridomas derived from (B6xA)F1 T cell lines specific for the three species variants of myoglobin amino acids 67-79, A) sperm whale B) equine and C) mouse. The chosen T cell lines were sperm whale T cell line “Sp”, equine T cell line “Eq” and mouse T cell line “Mu#2” from Figure 3. The Vβ chain was determined by screening the T cell hybridoma cDNA with a set of Vβ-specific 5’ primers (as described in M&M) and the result is shown in the third column. The CDR3 amino acid sequence (column IV) as well as the Jβ usage (column V) were determined for most of the T cell hybridomas. Boxes in column IV separate Vβ and Jβ sequences from Dβ sequences and N-region additions (in bolded letters). The cross-reactivity of each T cell hybridoma for the three species variants of myoglobin 67-79 was determined in a lymphokine secretion assay (as described in M&M) and is shown in column IV. The response to 10 μM myoglobin peptide was estimated as follows: “+++” for cpm > 100,000; “++” for cpm 50,000-99,999; “+” for cpm 10,000-49,999; “+/-” for cpm < 9,999 and “-” for cpm equal to response without Ag. The number of T cell hybridomas expressing a particular TCR sequence is shown in column II.

* n.d: non determined

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Figure 4. T cell repertoire analysis of T cell hybridomas derived from SMW-, EqM- and MM 67-79-specific T cell lines.
Figure 5. Intracellular cytokine staining of (B6xA)F1 T cell lines responsive to species variants of myoglobin determinant 67-79.

Three independent MM 67-79-specific T cell lines, Mu#1, Mu#2 and Mu#3 (top three panels), one SWM 67-79- (Sp) and one EqM 67-79- (Eq) specific T cell lines (bottom two panels) were tested for cytokine production by intracellular staining and analyzed by FACS (as described in M&M). The cytokine response was first assayed after the 4th restimulation of the T cell lines, and did not vary over at least five subsequent restimulations. IFN-γ staining appears on the y axis and IL-4 staining appears on the x axis of each FACS panel. Percentages of cells contained within gated populations are displayed inside each gate. The numbers appearing in parenthesis refer to percentages of cells contained in the same gates, when IL-10 instead of IL-4 staining was displayed on the x axis. Isotype control stainings revealed < 1% cells in both FITC and PE channels.
CHAPTER IV

A New Look at MHC and Autoimmune Disease

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Reprint from: Science (1999), 284, 749-51
The strong genetic association that exists between certain autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and insulin dependent diabetes mellitus (IDDM), and the expression by the patient of particular major histocompatibility complex (MHC) haplotypes (the region of the genome encoding this linked series of genes), has been known for over two decades (Svejgaard et al., 1983). The current explanation for this association between MHC and autoimmune disease proposes that disease associated MHC molecules efficiently bind autoantigens involved in the pathophysiology of the disease, leading to a peripheral T cell mediated immune response to the autoantigen and resultant autoimmune sequelae (e.g. arthritis, demyelination, or pancreatic beta cell destruction) representing the particular disease. However, recent results in an animal model of autoimmune diabetes, the nonobese diabetic (NOD) mouse, have allowed the generation of a new hypothesis to explain the role of MHC in autoimmunity. This hypothesis suggests that the genetic association between MHC and autoimmune disease results from “altered” thymic selection in which high affinity self-reactive (potentially autoreactive) T cells escape thymic selection (self-peptides bound to specialized thymic antigen-presenting cells shape, or select, the T cell repertoire) as a result of the poor self-peptide binding properties of the disease associated MHC Class II molecules (Ridgway et al., 1998). This model offers an explanation for the unusual requirement of homozygous MHC class II expression for IDDM in NOD mice (and in human IDDM).

MHC molecules function by binding peptides for presentation to antigen-specific T cell receptors (TCRs) on T lymphocytes. The TCRs recognize MHC/peptide complexes on the surface of antigen-presenting cells (APCs). Our current understanding of MHC molecular function stresses two physiological roles for MHC class II gene products: 1) selection of the T cell repertoire in the thymus, and 2) presentation of foreign antigens in the periphery. By processing and presenting self-peptides bound to MHC molecules to developing thymocytes, thymic APCs first select the potential peripheral T cell repertoire (positive selection) and then purge this positively selected repertoire of T cells which react too strongly (negative selection) to self-peptide/MHC complexes. Less than 1% of pre-T cells entering the thymus survive these selection processes (Jameson et al., 1995). Subsequently, by presenting foreign peptides to the peripheral T cells that have survived this selection process in the thymus, peripheral APCs, expressing the identical MHC molecules as their thymic counterparts, initiate protective immune responses. This “dual role” of MHC class II molecules has contributed to our difficulty in deciphering their role in autoimmune disease, because the response, or lack of response, of the peripheral T cell population to any antigen is determined both by MHC-mediated thymic selection events as well as by the capacity of the MHC molecules to bind and present foreign antigen to peripheral T cells. An hypothesis accepted by most investigators is that autoimmune diseases are mediated by “pathologic” T cells, which
“inappropriately” recognize and respond to self-peptide/MHC complexes, possibly following activation by molecular mimics. It has been difficult to determine, however, whether the MHC effect, which allows T cells to mediate autoimmune attack lies at the central (thymic) or peripheral level, or both. Another complexity in understanding the role of MHC in genetic predisposition to autoimmune disease results from the fact that autoimmune diseases are multi-genic diseases. Thus, the MHC acts in conjunction with multiple other genes, none of which are themselves necessary and sufficient, to produce autoimmunity.

Recent results obtained in studies in the NOD model of autoimmune diabetes have begun to unravel these complex problems. NOD mice spontaneously develop an autoimmune syndrome in which autoreactive CD4+ T cells infiltrate multiple organs, including the pancreas. Several reports have described autoreactive T cells in NOD mice that recognize multiple pancreatic and non-pancreatic self-peptides and are capable of transferring at least three distinct autoimmune syndromes to naive recipients (Costagliola et al., 1996; Humphreys-Beher et al., 1994; Kaufman et al., 1993; Tisch et al., 1993). Our lab demonstrated that self-tolerance of NOD mice could be “broken” by immunization with self-peptides (Ridgway et al., 1996). The immunized NOD mice developed autoreactive CD4+ MHC class II restricted T cells that recognized endogenously processed and presented self-peptides, identical to the self-peptides used for immunization. However, NOD mice demonstrated normal T cell responses following immunization with foreign antigens. This suggested that there was not a generalized “hyper-responsive” T cell population in NOD mice; rather an effect manifest by the ability of NOD T cells to respond “inappropriately” to self antigens in the periphery (Ridgway et al., 1996). The bias of the NOD peripheral T cell repertoire towards autoreactivity following immunization with self-peptides, while maintaining a normal T cell response to foreign antigens, suggested that the MHC effect in NOD autoimmunity might be at the level of thymic selection of the T cell repertoire.

Further insights resulted from the biochemical characterization of the NOD MHC class II molecule. Comparison of the MHC class II sequences from diabetic humans and NOD mice originally revealed a fundamental structural similarity: non-Asp amino acids at position 57 of an MHC class II β chain (Acha-Orbea and McDevitt, 1987). Mice and humans with homozygous expression of non-Asp at β chain position 57 demonstrated increased susceptibility to diabetes, but the incidence of diabetes dropped dramatically in heterozygotes that expressed only one copy of the disease associated allele (Makino et al., 1985; Prochazka et al., 1987; Wicker et al., 1987). While over 80% of female NOD mice develop disease, only a small percentage of NOD F1 mice (1-3%) developed diabetes (Wicker et al., 1987). How could a two-fold reduction in cell surface expression of the class II protein result in a thirty-fold (or greater) decrease in disease incidence, if I-A^g^ functioned solely as an efficient binder of diabetogenic peptides? Unanue et al. approached
this question by investigating the biochemical characteristics of the NOD class II molecule, I-A<sup>g7</sup>. This analysis of I-A<sup>g7</sup> demonstrated the exact opposite peptide binding characteristics to those predicted by the good peptide-binding model of MHC. Rather than being an efficient binder of antigens, I-A<sup>g7</sup> was found to be a poor peptide binder, and to demonstrate structural instability (Carrasco-Marin et al., 1996).

The basis for the new model of MHC association with autoimmunity comes in part from these findings of biochemical defects of I-A<sup>g7</sup>, demonstrated by Carrasco-Marin et al. (Carrasco-Marin et al., 1996), as well as from our findings of the unusual NOD autoreactive T cell responses (Ridgway et al., 1996; Ridgway et al., 1998). Combined, these studies suggest that, in an avidity model of thymic selection (Ashton-Rickardt et al., 1994; Kim et al., 1996; Sebzda et al., 1994), unstable I-A<sup>g7</sup> could produce an effectively decreased [MHC: peptide] concentration on thymic APCs, which would select a population of T cells with increased TCR affinity to attain the avidity threshold required in thymic positive selection (Fig. 1). These high affinity self-reactive T cells would enter the periphery and, in collaboration with multiple other disease related genes, mediate autoimmunity once an inflammatory event "broke" self-tolerance. Recent results from several labs have supported this hypothesis. Kanagawa et al. found, by limiting dilution analysis, that

I-A<sup>g7</sup> was associated with quantitatively increased T cell autoreactivity in the peripheral T cell population (Kanagawa et al., 1998). We demonstrated that I-A<sup>g7</sup> allowed thymic selection of autoreactive T cells, while imposition of a single copy of a second, conventional, MHC class II molecule, I-A<sup>4</sup>, (with all other non-MHC NOD genes held constant), eliminated these autoreactive T cells from the periphery, thus explaining the effect of MHC heterozygosity on decreased frequency of IDDM as the result of T cell thymic selection events (Ridgway and Fathman, 1998; Ridgway et al., 1998; Ridgway et al., 1998). These combined results suggest that

I-A<sup>g7</sup> acts in the thymus of NOD mice to allow selection of a T cell population with an increased mean affinity for self-peptide/MHC. The resultant increased "strength of signal" generated by these "high affinity TCRs" following activation by non-self and subsequent encounter with self antigen, may explain the broad range of T cell autoreactivity found in these mice.

This model of defective thymic selection by homozygous NOD I-A<sup>g7</sup> is similar to results obtained in several recent knockout and transgenic mouse models. Laufer et al. expressed MHC class II exclusively in the thymic cortical epithelium of MHC class II knockout mice, and demonstrated large numbers (up to 5% of total lymphocytes) of self-reactive T cells which "autoproliferated" to syngeneic spleen cells, representing "unopposed positive selection" (Laufer et al., 1996). Several groups knocked out H2-M, which functions in loading a diverse peptide
repertoire into MHC class II molecules. The T cells from these mice underwent positive selection, but demonstrated “autoproliferative”-like responses to wild-type APCs, again suggesting defective negative selection in the setting of efficient positive selection (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). In a transgenic model, Schmidt et al. showed that diabetogenic T cells could be deleted by the disease-resistant MHC molecule, lending further support for function of MHC at the level of repertoire selection (Schmidt et al., 1997). Clearly, reducing the problem of the genetic association of MHC and autoimmunity from the level of the whole organism (i.e. disease readout) to the level of MHC class II biochemistry and MHC controlled T cell repertoire selection has been a fruitful approach. If the association of MHC with autoimmunity can be ascribed to thymic selection of the peripheral TCR repertoire, the function of other genes required in the pathologic processes of autoimmune inflammation can be more easily studied.

ACKNOWLEDGMENTS

We thank J. Rothbard, D. Kim, and L. Wicker for helpful discussions, K. Lejon and A. Ridgway for critical reading of the text, C. Taylor for technical assistance, and R. Kizer for her secretarial assistance.
Figure 1. An avidity model for thymic selection in NOD mice.

The components in bold letters (TCR affinity and MHC stability) are operative in NOD mice. The critical assumption in this model is that the avidity necessary to attain positive and negative thymocyte selection thresholds \([A(p,n)]\) is constant between mouse strains, reflecting intrinsic thymocyte signaling. Additive contributions to the avidity \((x)\) might differ between NOD and other mouse strains; however, studies with MHC congenic NOD mice suggest they are not critical in NOD repertoire selection (see text). Given these constraints, a global decrease in [I-Ag7 MHC: peptide] stability would necessitate a compensatory increase in the mean population TCR affinity to attain the constant avidity thresholds of positive and negative selection. The result would be a NOD peripheral T cell repertoire biased towards TCR sequences with greater intrinsic affinity towards self-MHC: peptide. An MHC molecule without global biochemical defects could bind a limited number of self-peptides poorly, producing a limited set of higher affinity T cells (Liu et al., 1995).
Unresponsiveness to self is maintained through two mechanisms of immune regulation: thymic-negative selection and peripheral tolerance. Although thymic-negative selection is a major mechanism to eliminate self-reactive T cells, normal mice have readily detectable populations of T cells reactive to self-proteins but do not exhibit autoimmune responses. It has been postulated that autoimmune disease results from breakdown or loss of peripheral tolerance. We present data that demonstrate that peripheral tolerance or unresponsiveness to self can be broken in nonobese diabetic (NOD) mice. Immunization of NOD mice (but not of conventional mice) with self-peptides caused an immune response to self-peptide with resultant autoprofiferation of peripheral lymphocytes. Autoproliferation of self-reactive T cells in NOD mice resulted from the recognition and proliferation of the activated T cells to endogenously processed and presented self-antigens. This loss of self-tolerance demonstrated in vitro may well be the basis of NOD autoimmune disease in vivo.

Analysis of the Role of Variation of Major Histocompatibility Complex Class II Expression on Nonobese Diabetic (NOD) Peripheral T cell Response

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The current paradigm of major histocompatibility complex (MHC) and disease association suggests that efficient binding of autoantigens by disease-associated MHC molecules leads to a T cell-mediated immune response and resultant autoimmune sequelae. The data presented below offer a different model for this association of MHC with autoimmune diabetes. We used several mouse lines expressing different levels of I-Ag7 and I-Ak on the nonobese diabetic (NOD) background to evaluate the role of MHC class II in the previously described NOD T cell autoproliferation. The ratio of I-Ag7 to I-Ak expression correlated with the peripheral T cell autoproliferative phenotype in the mice studied. T cells from the NOD, [NOD x NOD. I-Anull]F1, and NOD I-Ak transgenic mice demonstrated autoproliferative responses (after priming with self-peptides), whereas the NOD.H2(h4) (containing I-Ak) congenic and [NOD x NOD. H2(h4) congenic]F1 mice did not. Analysis of CD4(+) NOD I-Ak transgenic primed lymph node cells showed that autoreactive CD4(+) T cells in the NOD I-Ak transgenic mice were restricted exclusively by I-Ag7. Considered in the context of the avidity theory of T cell activation and selection, the reported poor peptide binding capacity of NOD I-Ag7 suggested a new hypothesis to explain the effects of MHC class II expression on the peripheral autoimmune repertoire in NOD mice. This new explanation suggests that the association of MHC with diabetes results from "altered" thymic selection in which high affinity self-reactive (potentially autoreactive) T cells escape negative selection. This model offers an explanation for the requirement of homozygous MHC class II expression in NOD mice (and in humans) in susceptibility to insulin-dependent diabetes mellitus.

GENERAL DISCUSSION AND OUTLOOK

Finding new means to track antigen-responsive T cells during the course of an immune response \textit{in vivo} is essential to the study of lymphocyte differentiation, in particular the search for approaches which avoid the use of TCR-transgenic T cells and which are not confined to responses dominated by restricted TCRs. For many years, TCR transgenic animals have been used as the major source of antigen-specific T cells for the study of T cell immune responses both \textit{in vitro} and \textit{in vivo}. However, this approach has not allowed investigators to address questions of T cell repertoire diversity, clonal/affinity maturation, and TCR repertoire development. Recently, a few methods have been proposed to visualize antigen-specific T cells during an immune response \textit{in vivo} in normal animals, but unfortunately, they are only applicable to the study of responses dominated by a single TCR V beta/alpha combination.

An alternative to these approaches is presented in this thesis. Chapter I describes the identification \textit{in vivo} of activated antigen-specific CD4$^+$ T cells based on their up-regulated expression of cell surface CD4. The direct isolation and characterization of antigen-specific CD4$^{{\text{high}}}$ T cells from the draining lymph nodes of SWM 110-121 immunized DBA/2 mice at various time points following primary and secondary immunization, shed new light on the development of Th-mediated responses in normal animals as reported in Chapter II and III.

First, evidence provided in Chapter II supports a T cell affinity selection mechanism acting early during the CD4$^+$ T cell immune response \textit{in vivo}. At three days after primary immunization, there is a diverse antigen-specific CD4$^+$ T cell repertoire, while by 6 days after primary immunization with SWM 110-121, T cell subsets with intermediate affinity for the antigen and expressing canonical CDR3 sequences in their TCRs had been selected for expansion from the much larger set of antigen-reactive cells. The early expansion and persistence of intermediate affinity T cells during the course of the secondary immune response suggested that this process must be central in shaping CD4$^+$ T cell memory. Unlike the extensive and elaborate affinity maturation processes characteristic of the development of B cell memory, the affinity selection observed in the T cell compartment appears to be a minor process of moderate amplitude. Nevertheless, despite similarities in their general structure, antibodies and TCRs use very disparate strategies of antigen recognition. While antibodies recognize and bind antigens in their tertiary configuration when present in the blood and tissue fluids, TCRs recognize linear peptide determinants complexed within the peptide binding cleft of MHC molecules during a cell-cell contact. Furthermore, TCRs bind their ligands with inherently lower affinities than antibodies (dissociation constants ranging between $10^{-4}$ and $10^{-7}$ M for TCRs, compared to $<10^{-9}$ M for most Igs).
These transient interactions, caused by the low affinity recognition of the TCR for its ligand, are an essential feature in recent proposed models of T cell activation like the kinetic proof reading (McKeithan, 1995; Rabinowitz et al., 1996) and serial TCR triggering models (Valitutti et al., 1995). Both models explain how a low affinity TCR can distinguish between small differences in the antigen and how the TCR can respond in an on and off fashion to antigenic stimuli within a very narrow affinity range. McKeithan and Rabinowitz’s kinetic proof reading model predicts that interactions within a narrow range of TCR affinities, which can sustain signaling over a determined threshold, will lead to optimal responses. This model is based on the observation that T cell activation requires the assembly, to the phosphorylated TCR, of multiple layers of proteins, suggesting that certain thresholds of strength and duration need to be exceeded in order to assemble the correct signaling complex required for an “optimal” response. Lanzavecchia and Valitutti’s serial triggering model also explains how slight changes in on- and off-rates can result in distinct T cell responses, but unlike the kinetic proof reading model, it proposes that an “optimal” on- and off-rate combination in the TCR-ligand interaction will result in optimal activation of the T cell, while any deviation from this optimum will lead to a normal distribution of responses, from weak activation to partial activation to antagonism to no effect at all.

While the serial triggering model might explain why in our system selection for T cell subsets with intermediate affinities for the antigen was observed in the first 6 days of the immune response, it does not explain why at day 3 after immunization, T cells displaying higher affinities than the proposed “intermediate optimum” proliferated better than the intermediate affinity T cells. The kinetic proof reading model, conversely, can explain why higher affinity T cells will result in a more robust response, but does not explain why intermediate affinities were favored for expansion in vivo. A possible explanation is that too strong an interaction would be detrimental, as evidence has demonstrated that it leads to death by apoptosis of the T cell.

In Chapter III of this thesis, using the same antigen model as in Chapter II, a direct correlation was shown between the structure and affinity of the TCR for the [peptide:MHC] complex and Th effector differentiation in vivo, whereby low affinity T cells expressing non-canonical TCRs developed Th2-like responses, and T cells expressing canonical TCRs and displaying intermediate and high affinities for the antigen developed Th1-like responses. These results suggested that the Th differentiation of the DBA/2 response to SWM 110-121 towards a Th1 phenotype in vivo, was the result of the same T cell affinity selection processes described above, which, as a result of promoting the selective expansion of T cells with intermediary affinities for antigen, i.e., T cells with “optimal kinetics” for antigen recognition, simultaneously skewed the T cell response towards a Th1 phenotype (Fig. 1).
Studies of Th differentiation performed in vitro, using T cells from mice expressing transgenic antigen receptors of known specificities have shown that in the absence of exogenously added cytokines, naive TCR transgenic T cells will naturally differentiate towards one Th subset. Our results suggest that it is the strength of signal received by the TCR which will determine which differentiation pathway the T cell follows, if all other variables were held constant. According to the kinetic proofreading model of T cell activation, the length of TCR occupancy, itself determined by both off rate and affinity for the [peptide:MHC] complex, determines the completion of assembling of the signaling complex necessary for full activation of the cell. Short-lived occupancy, as in case of a low affinity interaction, will stimulate the TCR but will be unable to sustain signaling long enough to generate the complete signaling complex. It has been reported that less than optimal interaction with APLs will lead to incomplete ζ chain phosphorylation resulting for example in IL-4 production in the absence of proliferation (Evavold and Allen, 1991; Kersh et al., 1998; Sloan-Lancaster et al., 1994; Sloan-Lancaster et al., 1994). The induction of Th1 differentiation as the default pathway of Th phenotype development might only occur under optimal kinetics of T cell-antigen recognition. Less than optimal kinetics, by causing only a transient pattern of signal transduction, might prevent the T cell from undergoing Th1 differentiation, and result in Th2 differentiation.

Currently, a central role has been attributed to the cytokines present at the initiation of the immune response in determining Th1 or Th2 differentiation in vivo. The finding, that the strength of the TCR:[peptide:MHC] interaction alone could directly determine the differentiation pathway of a T cell, raised the question as to the hierarchy of events and factors influencing Th differentiation. Will exogenous factors, like IL-4 and IL-12 cytokines present at the initiation of the immune response merely enhance or decrease an already predetermined process of Th-differentiation based upon the avidity of TCR recognition of the antigen, or will cytokines actually modulate the direction of Th differentiation regardless of TCR:[peptide:MHC] avidity? Current evidence from in vitro studies would favor the latter, since naive T cells from TCR transgenic mice can be driven to either one Th subset by exogenously adding IL-12 or IL-4 to the cultures. However, results presented in this thesis seem to favor the former situation, since in our system low affinity T cells still differentiated into Th2 effectors, although immunization was performed in CFA (which is believed promote Th1 differentiation by inducing IL-12 production by macrophages). Further studies will be required to define the hierarchical contribution of signaling through the TCR versus cytokine receptors in Th differentiation in vivo.

The role of TCR affinity in determining Th1 or Th2 differentiation was further demonstrated in the (B6xA) F1 response to species variants of myoglobin determinant 67-79. T cell repertoire analysis demonstrated that distinct T cell repertoires are involved in (B6xA) F1
mice in the response to self versus foreign myoglobin 67-79. Either as a result of negative (or lack of positive) selection in the thymus, only a low affinity T cell repertoire was able to respond to the self myoglobin determinant. The low affinity response to MM 67-79 resulted in Th2 differentiation, while the high affinity repertoires specific to foreign versions of myoglobin 67-79 lead to Th1 differentiation of the immune response. The finding in this system, that the Th1-cell selection process was limited by the overall affinity of the pre-existing T cell repertoire was a demonstration of the role of central (thymic) selection in determining the development of Th1 or Th2 responses in vivo. By determining the range of affinities of the selected peripheral T cell repertoire for any specific antigen, thymic selection would control the overall avidity of certain TCR:[peptide:MHC] interactions, resulting in the predisposition to the development of either a Th1 or a Th2 response following immunization with antigen (Fig. 2). Some reports have suggested a role for genetic background in directing Th subset development in vivo, without providing a mechanism (Hsieh et al., 1995; Murray et al., 1992). It might be interesting to go back to these studies to determine whether genetic predisposition towards Th1 or Th2 immunity might have been linked to a central effect of thymic selection by MHC.

The genetic predisposition towards either Th1 or Th2- dominated responses can be decisive in the response to pathogens but can also have important consequences in the development of autoimmune responses. In chronic autoimmune diseases, pathogenic roles have been attributed to Th1 cells, while Th2 cells can confer protection. Low affinity interactions of self-reactive T cell repertoires with self-antigens in the periphery resulting into Th2 responses could therefore constitute an important general peripheral regulatory mechanism against autoimmunity.

Our studies in Chapter IV, of the development of self versus foreign specific Th-mediated immune responses in the NOD mouse, have lead to a new model of MHC and disease association. In this model, altered thymic selection in the NOD mouse caused by the unstable MHC class II molecule I-A^d, resulted in the escape in the periphery of a high affinity self-reactive TCR repertoire, which in collaboration with multiple other disease related genes, might mediate autoimmunity once an inflammatory event breaks self-tolerance. If this model is correct, NOD mice should have a higher affinity population of the selected self-reactive T cell repertoire compared to a normal mouse strain. Supporting this, preliminary data show that NOD mice develop high affinity, Th1 responses to both foreign and self peptides (while normal mice develop Th2 responses to self-proteins), suggesting that disruption of self-tolerance in NOD mice may unmask functional loss of regulation at two levels: 1) the presence of a T cell repertoire enriched in high affinity autoreactive T cells, and 2), as a consequence of increased TCR affinity in T cells reactive to self, a Th1 response to self-peptides.
The functional loss of regulation described in the NOD mice could also provide an explanation for susceptibility of autoimmune mice models to molecular mimicry. While in normal mice strains, high affinity Th1 responses are seen to foreign antigens in an inflammatory context, once activated, the cells will cross-react with self-antigens with low affinity. Conversely, in NOD mice, escape of high affinity autoreactive cells from thymic selection might simultaneously produce a population of cells which can induce an inflammatory, Th1 response to self when activated in the periphery by cross-reactive foreign epitopes, leading to the autoimmune sequelae.

In conclusion, this work offers a novel understanding of the development of self versus foreign specific Th immune responses in both normal and autoimmune disease models and suggests that normal thymic selection might allow dual protection against autoimmunity. First, by only allowing T cells with low affinity to self to escape thymic selection and second, by predetermining the production of “regulatory” Th2 like cytokines in T cells reacting with low affinity interaction to self-peptides in the periphery.
Figure 1. A Th1/Th2 cell selection process occurs during the DBA/2 response to SWM 110-121 in vivo.

Early in the response to SWM 110-121, a diverse repertoire of T cell displaying a whole range of TCR affinities for the antigen is activated, whereby T cell expressing low affinity, non-canonical TCRs differentiate into Th2, while T cells expressing higher affinity canonical* TCRs develop into Th1 effector cells. Soon, however, affinity selection will promote the selective expansion of T cells with intermediate affinities for the antigen, such that by day 6, Th1 cell expressing the canonical TCR will dominate the response, while the Th2 response component of the response will disappear.

*Canonical TCRs contain the amino acid sequence (A/G)WDWx(x) the CDR3 region of their Vβ8 chain.
TCR avidity for [self-antigens : MHC] complexes in the thymus:
- high
- low

Overall TCR affinity of selected T cell repertoire for the [immunizing peptide : MHC] complex in the periphery:
- high for [SWM 67-79 : MHC] or [EqM 67-79 : MHC]
- low for [MM 67-79 : MHC]

Figure 2. The Th1/Th2 cell selection process is limited by the overall affinity of the pre-existing T cell repertoire.

In normal mice, thymic selection in the thymus will shape the peripheral T cell repertoire by selecting T cells which can engage the [self-peptides:MHC] complexes with low avidity.

Immunization in CFA was performed with both foreign (right side) and self (left side) variants of myoglobin determinant 67-79. In a system where all environmental factors including [antigen:MHC] density were kept constant and where the only variable affecting the commitment to Th phenotype consisted of the selective activation of a self- or foreign-reactive peripheral T cell repertoire only the high affinity foreign-reactive T cell repertoires lead to Th1 responses in vivo, while the low affinity, self-reactive T cell repertoire resulted in the development of a Th2 response.

We conclude that T cells, which at the given ligand density could have potentially engaged self-myoglobin with such an avidity to promote a Th1 response must have not been present in (B6xA)F1 mice, due to negative or lack of positive selection in the thymus.
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### Abbreviations

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<td>Ab</td>
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<td>Ag</td>
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<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HuCIV</td>
<td>human collagen IV</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>IFA</td>
<td>incomplete Freund's adjuvant</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ITAM</td>
<td>CD3 immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>Jβ</td>
<td>junctional region beta</td>
</tr>
<tr>
<td>LDA</td>
<td>limiting dilution analysis</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MCC</td>
<td>moth cytochrome c</td>
</tr>
<tr>
<td>2-ME</td>
<td>β₂-Mercaptoethanol</td>
</tr>
<tr>
<td>MHC I or II</td>
<td>major histocompatibility complex class I or II</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>MM</td>
<td>mouse myoglobin</td>
</tr>
<tr>
<td>NOD</td>
<td>nonobese diabetic mouse</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCC</td>
<td>pigeon cytochrome c</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
</tr>
<tr>
<td>SWM</td>
<td>sperm whale myoglobin</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TGF-β</td>
<td>tumor growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Vβ</td>
<td>variable region beta</td>
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</table>
CURRICULUM VITAE

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Fellowship from the Foundation of the Schweizerischen Verband der Akademikerinnen (SSVA)

1996-1998 Fellowship from the ETH-Stipendiendienst
ACKNOWLEDGMENTS

My first thank you goes to Hans Hengartner for the trust he placed in me in agreeing to be my thesis advisor while completing my experimental work at Stanford University. I am thankful for his help in directing me to the right laboratory and in helping to organize the funds necessary to support me during my stay abroad. I am grateful for his patience and availability throughout my graduate work, especially my “after hours” long distance calls and my short visits to Switzerland with little advance warning.

There are no limits to my gratitude for my mentors and advisors Garry Fathman and Bill Ridgway. I am very grateful to Garry for his excellent scientific guidance, his contagious enthusiasm and optimism, his infinite understanding and immense support during professional - and also less professional - crises, and for his immeasurable generosity. Garry’s infectious love for science has been a driving force in the lab and I can not thank him enough for making these past years a phenomenal and unforgettable experience.

I am extremely thankful to Bill for his excellent teaching and mentoring, for the countless exciting scientific discussions and critical advice. I want to thank him for “strongly suggesting” that I stop doing experiments when it was time to analyze the data or to write the paper, and his patience when I consistently would not listen. On a more personal note, I thank Bill for his friendship and the enjoyable and amusing company in and outside the lab, from the famous philosophical discussions in the tissue culture room, to the unforgettable karaoke at the workshop in Italy.

I am especially grateful to my graduate student colleague and friend Dewey Kim for the countless constructive discussions and critiques of my project, for his ability at any time of day or night to find *the* flaw in my critical thinking, for his readiness to help with any kind of scientific and non-scientific problem, for his constant moral and emotional support and for contributing to a fun and exciting time both in and outside the lab.

I wish to thank my student Niroshana Anandasabapathy not just for her excellent work and enjoyable company but also for her innumerable (futile) efforts to teach me about political correctness and her constant and healthy reminders about lunch.

I am thankful to Debra Bloom for teaching me the art of culturing T cell clones and T cell hybridomas and for introducing me to TCR sequencing.

I want to thank all the Ph.D. students and Post-docs of the lab for creating a friendly and collaborative atmosphere; in particular, Irene Ruiz for her refreshing presence in the lab and her friendship and generosity in introducing me to some of the best aspects of Californian life.

Many thanks to Cariel Taylor and Violette Paragas for their excellent technical assistance and to Robyn Kizer and Elisabeth Hoernhager for an impeccable secretarial support.

I truly appreciated the support of Claire Holness, Dirk Kreder, Craig Estes, and Ralf Schwandner during the writing of this thesis.

I would like to thank the Stipendiendienst of the ETH for its generous financial support.

I sincerely wish to thank my Mother for all the meals she has tirelessly cooked and prepared for me to pick up on my way to work, and both of my Parents for their love and spiritual support throughout my entire doctoral work.

Last but not least, I am extremely grateful to Dirk Brockstedt who with constant loving support and inexplicable patience, has not only helped me to maintain a healthy balance between home and work, but through innumerable scientific discussions has immensely contributed the successful completion of this thesis.