Insights into the dynamics of T cell clonal expansion and the functional heterogeneity of memory CD4 T lymphocytes using superantigens

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Abstract

Superantigens trigger the polyclonal activation of human T cells. We exploited this property to gain insight into the mechanisms governing CD4 T cell expansion and to study the functional heterogeneity of naïve and memory CD4 T cell subsets. We show that the amount of TCR ligand affects the evolution of a T cell response in two ways: by shaping the diversity of the T cell population recruited in the proliferative pool and by affecting the progression of these precursors into cell cycle. These two processes characterize a hierarchy of recruitment of cells that strongly correlates with the efficiency of TCR engagement but not with the relative precursor frequency in the non-immune repertoire. Remarkably, once established, the distribution of T cell clones within a selected repertoire is maintained by the characteristic of T cells to expand at a rate that is independent of quantitative differences in ligand exposure. Moreover, at optimal ligand concentrations that lead to the simultaneous expansion of all responsive T cell clones, we observed a marked clone-specific heterogeneity in the capacity to secrete cytokines. The functional heterogeneity of different CD4+ T cell subpopulations was also studied using a superantigen model. Based on the expression of CD45RA and CCR7, four distinct subsets of CD4+ T lymphocytes can be identified: naïve (CD45RA+ CCR7+), central memory (T_{CM}, CD45RA- CCR7+), effector memory (T_{EM}, CD45RA- CCR7-) and a previously uncharacterized subset (CD45RA+ CCR7-). In CD8 T cells, this subset has been shown to comprise "terminally differentiated" effector memory cells. The four subsets show different functional sensitivities for the superantigens, the T_{EM} population being the most sensitive and the naïve cells being the least sensitive, as measured by the upregulation of activation markers. We show that the CD4+ CD45RA+CCR7- subpopulation, which is rarely detectable in healthy individuals, is enriched in T cells having strong immediate effector functions but impaired proliferative potential. By evaluating cytokine secretion, a functional heterogeneity between the subsets, as well as within a given subset, is demonstrated. These findings contribute to our understanding of T cell expansion and immunologic memory and may be relevant to the design and evaluation of vaccines.

Résumé

Les superantigènes provoquent une forte stimulation polyclonale des lymphocytes T humains. Nous avons exploité cette propriété afin d'étudier les mécanismes qui régulent l'expansion clonale des cellules T ainsi que l'hétérogénéité fonctionnelle des cellules T naives et mémoires. Nous démontrons que des doses sous-optimales de ligand affectent l'évolution de la réponse immunitaire de deux façons : en restreignant la diversité des lymphocytes T recrutés dans la réponse et en augmentant le temps requis pour l'expansion des cellules T recrutées. Les clones T préférentiellement sélectionnés à basse concentration de ligand sont ceux dont le RcT est le plus efficacement engagé, indépendemment de la fréquence de ces clones dans la population initiale. La dose de ligand affecte uniquement le délai avant la première division cellulaire, la vitesse de prolifération devenant indépendante de la dose initiale durant les divisions subséquentes. De plus, à des concentrations optimales de ligand qui causent l'expansion simultanée de tous les clones spécifiques, on observe néanmoins de grandes différences parmi ces clones dans la capacité de sécréter des cytokines. Nous avons aussi étudié l'hétérogénéité fonctionnelle des différentes populations de lymphocytes T CD4. Quatre sous-populations de lymphocytes T CD4 peuvent être identifiées selon l'expression d'isotypes de CD45 et du récepteur de chimiokines CCR7 : naïve (CD45RA+CCR7+), mémoire « centrale » (T_{CM}, CD45RA-CCR7+), mémoire « effectrice » (T_{EM}, CD45RA-CCR7-) ainsi qu'une sous-population non-caractérisée auparavant chez les CD4 (CD45RA+CCR7-). Chez les cellules T CD8, cette population a été associée à un stade avancé de différenciation. Des stimulations superantigéniques ont permis de démontrer que ces sous-populations possèdent des sensibilités différentes au ligand, les T_{EM} étant les plus sensibles et les cellules naives les moins sensibles. Nous démontrons que la sous-population CD4+ CD45RA+ CCR7-, qui est rarement retrouvée chez les sujets sains, est enrichie en cellules possédant de fortes fonctions effectrices mais ayant un potentiel prolifératif très réduit. La mesure de la sécrétion de cytokines nous a permis d'observer une hétérogénéité fonctionnelle entre les sous-populations et à l'intérieur d'une sous-population donnée. Ces travaux contribuent à améliorer la compréhension de l'expansion

clonale des cellules T et de la mémoire immunitaire et pourraient être pertinents pour le développement et l'évaluation de vaccins.

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P.M. Lavoie, A.R. Dumont and R.P. Sékaly originally conceived this project. H. McGrath performed preliminary experiments that provided optimal conditions for CFSE-tracking experiments. H. McGrath, P.M. Lavoie and A.R. Dumont performed the flow-cytometry analyses. P.M. Lavoie, A.R. Dumont and R.P. Sékaly primarily contributed to the interpretation of the results. P.M. Lavoie and A.R. Dumont made the figures. P.M. Lavoie, A.R. Dumont and R.P-Sékaly wrote the article.

Chapter 3: Functional heterogeneity of the human CD4 memory T cell subsets. Dumont, A.R. and Sékaly, R.P.

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Used, in part, throughout the introduction and discussion: Learning to remember: generation and maintenance of T-cell memory. Champagne, P*., Dumont, A. R.*, and Sekaly, R. P. DNA Cell Biol. 20[12].

* P. Champagne and A.R. Dumont contributed equally to this work.

Although this review article is not integrally incorporated in this thesis, passages from it were used to develop the thesis introduction, as well as part of the discussion. This

review article is the result of extensive brainstorming between the three authors, hypotheses and models having been developed through consensus and contribution of all involved. A. R. Dumont and P. Champagne made the figures and the article was jointly written. Reproduced with permission from Ann Liebert Publishing, Copyright (2001).

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1 Introduction

1.1 Overview of the immune system

1.1.1 General considerations

Throughout life, we are continuously exposed to a wide array of microorganisms, some of which are pathogenic and cause a threat to normal body function. Without efficient protective mechanisms, we would inevitably succumb to diseases caused by these pathogenic invaders. Immunity can be defined as the complex array of defensive measures developed by living organisms to protect themselves from invasion and colonization by foreign organisms (and from a dysregulation of the normal cell function in the case of cancer). The immune response can be divided into two closely interrelated processes: recognition and response. The hallmark of immune recognition is specificity. The immune system must therefore discriminate foreign molecules from the body's own components, whether proteins, carbohydrates, lipids or nucleic acids. Once recognition is achieved, the immune system triggers off a series of mechanisms involving different cell types and molecules to mount an effector response and eliminate or neutralize the invading organism.

1.1.2 Innate and acquired immunity

The immune system can be divided in two main functional branches: innate (non-specific) immunity and acquired (specific) immunity. Innate immunity is characterized by the lack of immunological memory; multiple encounters with the same antigen (Ag) do not result in a qualitatively or quantitatively enhanced response. Most pathogens encountered by an individual can be cleared within a few days by the innate defense mechanism. However, when a microorganism eludes the non-specific immunity, cells from the acquired immunity are activated and contribute to neutralizing the invading pathogen.

The first line of defense against microorganisms consists of the physical and anatomical barriers that prevent their entry inside the body. Intact skin not only prevents the entry of most pathogens but also inhibits bacterial growth due to its low pH (maintained at pH between 3 and 5 by the secretion of sebum by the sebaceous glands). The gastrointestinal, respiratory and urogenital tracts are composed of mucous membranes secreting viscous fluid called mucus that entraps foreign microorganisms; in the lower respiratory tract and the gastrointestinal tract, this mucus is excreted by the synchronous movement of cilia (peristalsis). Moreover, non-pathogenic organisms colonize the epithelial cells of mucous membranes (known as normal flora) and compete for attachment sites on epithelial cells and for essential nutrients.

The physiological barriers, including pH, oxygen tension and several soluble factors, constitute the second line of defense that contributes to innate immunity. For example, very few microorganisms can survive the acidic conditions in the stomach. The proteins (ex. lysosyme, lactoferrin) that compose some exocrine secretions like saliva, sebum, sweat and tears have antibacterial and/or antiviral properties and confer another level of protection. A very effective system of the innate immunity consists of the complement, a group of nearly 30 proteins which, upon activation by antigen-antibody complexes, interact in a regulated enzymatic cascade to generate a Membrane Attack Complex (MAC) able to lyse bacteria, viruses and infected cells (1).

Another level of protection is conferred by specialized cells of the innate immune system. Eosinophils kill surrounding pathogens by releasing reactive oxygen metabolites and cationic proteins, such as Major Basic Protein, into the extracellular environment (1). Basophils and mast cells express FcεRI, the high-affinity receptors for IgE, at their surface; cross-linking of FcεRI by antigen-bound IgE induces secretion of granules containing mediators like histamine, proteoglycans, proteases and cytokines as well as the synthesis of lipid mediators such as leukotrienes and prostaglandins (2). Professional phagocytes including monocytes, macrophages and neutrophils kill newly engulfed pathogens by production of reactive superoxide ions and induce pro-inflammatory signals by the secretion of cytokines and chemokines (3). Natural Killer (NK) cells destroy

infected and malignant cells that have lost the cell surface expression of Major Histocompatibility Complex (MHC) class I molecules; NK cells express killer-inhibitory receptors (KIR) which, upon binding to MHC class I, transmit an inhibition signal and block NK cells cytotoxic activity (4).

Innate immune response triggering normally relies on the recognition of pathogen-associated molecular patterns (PAMPs), which are conserved components of microorganisms (5). PAMPs include lipopolysaccharides (LPS), bacterial lipopeptides, peptidoglycan, flagellin, double stranded RNA (dsRNA), unmethylated bacterial DNA motifs (CpG DNA) (6). These PAMPS are recognized by a variety of receptors, the best known being the Toll-like receptors (TLRs) family; ten TLRs have been identified to date in humans and mice (6). The binding of TLRs to their specific ligands induces the expression, by activation of the NF-κB pathway, of host defense genes including cytokines, chemokines, antimicrobial peptides, MHC and co-stimulatory molecules (6).

An effective immune response relies on a concerted action of the innate and acquired branches of the immune system (7). The key cell type involved in the initiation and coordination of the immune response is the dendritic cell (DC). Immature dendritic cells, because they express high levels of most members of the TLR family, can recognize and internalize a wide variety of pathogens in the periphery; these events lead to the activation and maturation of the DCs (8). Mature DCs migrate to lymphoid organs, upregulate MHC, adhesion and co-stimulatory molecules cell surface expression and secrete high levels of Interleukin (IL)-12 (9). In the lymphoid organs, mature DCs orchestrate and/or modulate the activation of B and T lymphocytes, the two cell types mediating the acquired immune response (10). B lymphocytes are activated by soluble Ags, usually proteins in their native form, and migrate to specific areas of lymphoid organs called germinal centers where they undergo maturation (11;12). T lymphocytes generally recognized proteins that have been processed by the cellular machinery to generate short antigenic peptides presented in the context of MHC complexes (12).

1.1.3 Antigen processing and presentation: the Major Histocompatibility Complex

1.1.3.1 The MHC locus

The genes (more than 200) encoding the MHC are located on the short arm of the human chromosome 6 (6p21.3) and extend over 4 centimorgans of Deoxyribose Nucleic Acid (DNA) (figure 1). There are 3 families of MHC genes known as class I, II and III; in human, these genes are called Human Leukocyte Antigen (HLA). The MHC class I and II regions encode the cell surface glycoproteins responsible for peptide presentation to T lymphocytes as well as some proteins involved in Ag processing and presentation (13). The MHC class III region is defined as the segment between the class I and II regions and contains a heterologous collection of genes, some of them with known or potential immune/inflammatory function (13). For example, this region contains genes encoding for some of the complement system, as well as cytokines such as Tumor Necrosis Factor-α (TNF-α) and lymphokines A and B.

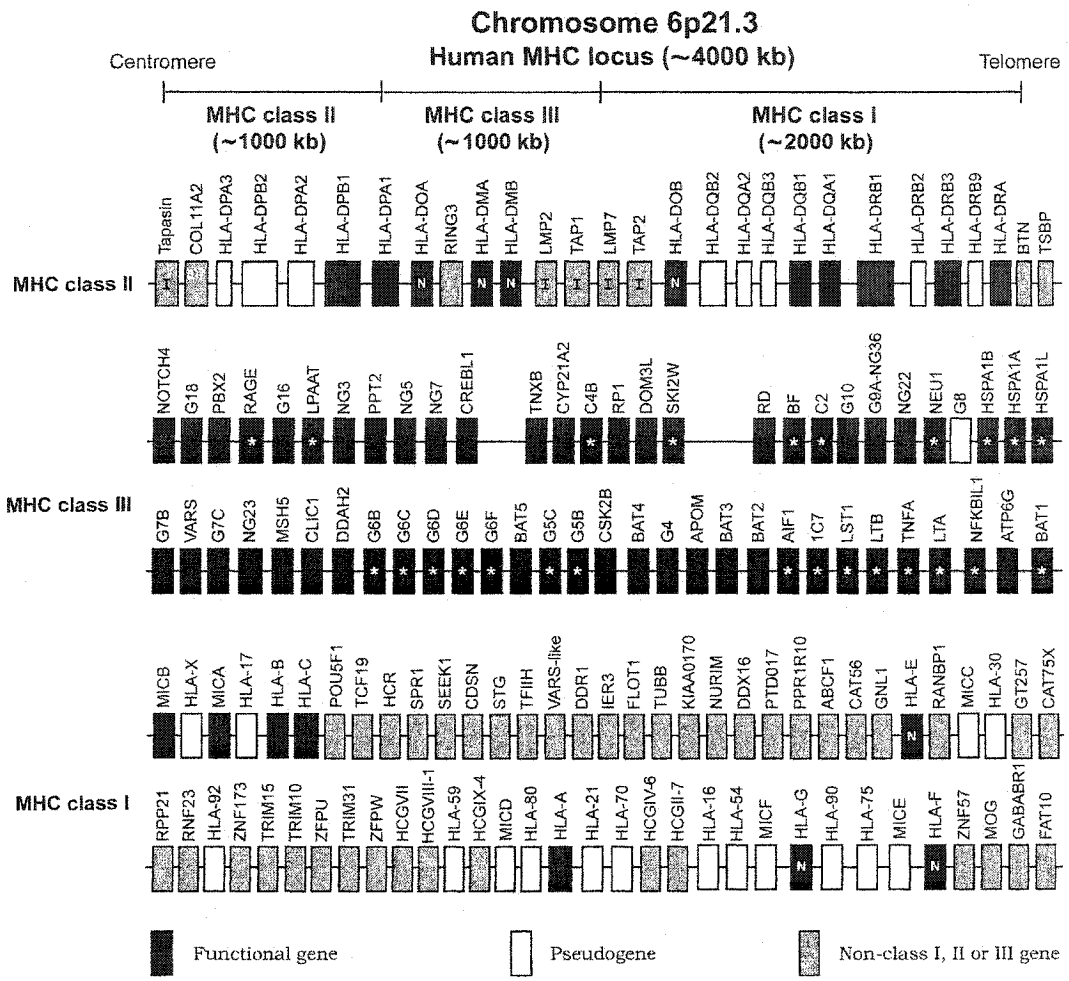

Figure 1. Genomic organization of the human MHC locus. *Genes involved in antigen processing and presentation in the MHC class I pathway are labelled by "I". Non-classical MHC molecules are labelled by "N". Genes with known or potential immune/inflammatory function are labelled by "*".*

1.1.3.2 MHC class I

The human MHC class I genes can be divided in two main classes : the highly polymorphic classical MHC class I (HLA-A, B and C) and the non-classical, less polymorphic MHC class I-like molecules (HLA-E, F, G and CD1). The classical MHC class I molecules are highly expressed at the surface of virtually all nucleated somatic cells and their primary function consists of presenting antigenic peptides to cytotoxic

CD8+ T cells. There is a high degree of polymorphism in classical MHC class I. Because both MHC class I α chain alleles of each locus are co-dominantly expressed, individuals homozygous for all three class I loci (HLA-A, B and C) express three different MHCs while individuals heterozygous for all loci can express six. Although present throughout the entire sequence of the MHC class I heavy chain, polymorphism is particularly concentrated in the residues making contact with the peptide, thereby influencing the array of peptides that can be presented and directly affecting the T cell response (14-16). The non-classical molecules have variable function, cell surface expression and tissue distribution. HLA-E and G bind to NK cells-specific receptors; this binding transmits a negative signal to the NK cell that inhibits the cytotoxic killing (17;18). The CD1 family includes 5 monomorphic and closely linked genes located on chromosome 1 (CD1a, b, c, d and e) (19). The CD1 molecules are specialized in presenting nonpeptide lipids and glycolipids to specific subsets of T lymphocytes (20-23).

MHC class I molecules consist of a heterodimeric, membrane-integrated glycoprotein formed by the non-covalent association between one of the polymorphic MHC class I α chain, a small monomorphic soluble protein called β_2-microglobulin (β_2m) and a short 8-10 amino acids peptide (24;25). The α chain is composed of three external domains (α1, α2 and α3) of approximately 90 amino acids, a 40 amino acids transmembrane domain and a cytoplasmic segment of 30 amino acids. The α3 domain and the β_2m adopt a structure that resembles that of an immunoglobulin domain. The membrane-distal α1 and α2 domains fold together to create a platform consisting of eight antiparallel β strands bordered by two α helices, thus forming a groove (or cleft) in which the antigenic peptide lies. This peptide binding groove places constraints on the size of the peptides that can be presented (usually 8 to 10 residues). Elution and identification of peptides bound to cell surface MHC class I molecules have shown that nonamers are favoured, suggesting that this length is more compatible with the size of the antigen-binding cleft (25-28). Moreover, some amino acids are highly conserved between peptides binding to a specific MHC class I allele; these anchor residues are usually hydrophobic and they interact with the amino acids in the peptide binding groove of the MHC class I molecules (25;28-32).

All nucleated cells of the human body display a set of about 10000 peptides bound to MHC class I molecules. In normal conditions, these peptides are generally derived from old or incorrectly folded cellular proteins that are ubiquitinylated and targeted for proteasome-mediated degradation in the cytoplasm (33;34). The 26S proteasomal complex consists of the 20S catalytic subunit, composed of four stacked rings of seven α-type and β-type subunits each forming a cylindrical structure, associated with fifteen regulatory subunits known as the 19S regulatory complex (35;36). The proteolytic activity responsible for protein cleavage is exerted by 3 of the β-subunits (b1, b3 and b5) which have trypsin-like, chymotrypsin-like and peptidyl-glutamylpeptide-hydrolysing specificities (37). In the context of an inflammatory process, the presence of specific cytokines like TNF-α and particularly Interferon (IFN)-γ induces the replacement of the three catalytic subunits of the proteasome by three homologous subunits called Low Molecular weight Protein (LMP)-2, LMP7 and Multicatalytic Endopeptidase Complex-Like (MECL)-1 to form a proteosomal complex referred to as the immunoproteasome (38). LMP2 and LMP7 (but not MECL1) are encoded by the HLA locus (39). IFN-γ also increases the expression of six new regulatory subunits, the proteasome-associated activator complex (PA)-28 which is required for immunoproteasome assembly and for efficient Ag processing (40;41). Overall, the incorporation of these four inducible complexes to form the immunoproteasome is believed to enhance Ag processing efficiency and to generate different set of peptides, presumably ones that are more favourable for Ag presentation (38;42). The peptides generated by the proteasome can be trimmed at their amino-terminal by cytoplasmic proteases (43;44). Interestingly, it has been shown recently that some antigenic peptides are not generated by the proteasome but rather by an aminopeptidase with endoproteolytic activity called cytosolic subtilisin-like tripeptidyl peptidase II (TPPII) (45;46).

Some of the peptides generated in the cytosol are subsequently picked up by molecules spanning the membrane of the Endoplasmic Reticulum (ER) called Transporter associated with Antigen Processing (TAP). The TAP heterodimeric complex is composed of two polymorphic subunits, TAP-1 and TAP-2, encoded by genes located in the MHC locus and is a member of the ATP-binding cassette (ABC) transporters superfamily (47-51).

Each TAP subunit has a N-terminal membrane spanning domain and a C-terminal ATPase domain (52). The TAP complex drives the translocation of the peptides from the cytoplasm to the ER lumen where the newly synthesized MHC class I molecules are waiting to be loaded with peptides. Following their entry into the ER lumen, some of the peptides can be further trimmed by specific proteases to generate 8-9 residues epitopes; one of them called Endoplasmic Reticulum Aminopeptidase associated with Antigen Processing (ERAAP) or Endoplasmic Reticulum Aminopeptidase 1 (ERAP1) has been recently identified by three different groups (53-56). The expression of this aminopeptidase is induced by IFN-γ (56). There is strong evidence that the presence of the appropriate MHC class I molecules is essential for this trimming (57;58).

In the ER lumen, the MHC class I heavy chain is bound to a chaperone called calnexin (59). Following the association of the heavy chain with β_2m, two other chaperones, calreticulin and tapasin, bind the newly formed heterodimeric complex (whether calnexin is able to bind the heterodimer is still not clear) (60-64). Tapasin physically links the TAP transporter and the complex containing calreticulin and the MHC class I, catalyzing the loading of the peptide in the MHC peptide binding groove (65;66). Tapasin-deficient mice have reduced MHC class I cell surface expression and impaired CD8 T cell development due to the absence of stably bound peptides to MHC class I upon exit of the complex from the ER (67). Tapasin interacts with the thiol-dependent oxydoreductase ERp57, which promotes disulfide bond isomerization in the MHC class I molecules; the abolishment of this interaction inhibits the loading of high affinity peptide in the groove and therefore form unstable cell surface MHC class I complexes (68). Finally, the peptide-loaded MHC class I molecules are transported from the ER through the Golgi complex to the plasma membrane (default secretory pathway) where they can interact with the T cell receptor (TCR) of the CD8+ T lymphocytes .

1.1.3.3 MHC class II

Like the MHC class I, the human MHC class II genes can be divided in two main classes: the highly polymorphic, classical MHC class II (HLA-DR, HLA-DP and HLA-DQ) and

the non-classical MHC class II (HLA-DM, HLA-DO), which show only limited polymorphism. Both the α and the β chains of each loci (HLA-DR, DP and DQ) exhibit high degree of polymorphism (except the monomorphic DR α chain) (39). Like the MHC class I, both alleles of each locus are co-dominantly expressed; fully heterozygous individuals express 6 different MHC class II molecules. The classical MHC class II molecules are expressed constitutively at the surface of dendritic cells, B cells and macrophages, collectively known as the "professional" antigen presenting cells (APCs) and their primary function consists of presenting antigenic peptides to CD4+ T cells. MHC class II proteins are expressed in other cell types either constitutively (in "nonprofessional" APCs like thymic medullary and cortical epithelial cells), following induction by IFN-γ (fibroblasts, mast cells and endothelial cells) or following activation (T lymphocytes) (69-71). The non-classical MHC class II molecules do not normally reside at the cell surface and they do not present antigenic peptides; their role is to modulate the binding of peptides to the classical MHC class II. HLA-DM is expressed by the same cell types as the classical MHC molecules while the expression of HLA-DO appears to be restricted to B lymphocytes, subset(s) of dendritic cells and to both cortical and medullary epithelial cells of the thymus (72;73).

MHC class II molecules are formed by the non-covalent association between two different polypeptides, a 33 kD α chain and a 28 kD β chain. Each chain contains a short cytoplasmic domain, a transmembrane domain and two extracellular domains (α1, α2, β1 and β2); the membrane-proximal α2 and β2 domains share sequence homology with the immunoglobulin-fold domain structure and the membrane-distal domains (α1 and β1) which form the peptide-binding pocket (74;75). This peptide-binding pocket is similar to that of MHC class I molecules, composed of a "floor" of eight antiparallel β strands and ridges formed by two α helices. Contrary to MHC class I structure, the peptide-binding cleft of MHC class II is open at both ends and consequently can accommodate peptides of variable lengths, ranging from 12 to 30 amino acids, with a preferred size of ~ 15 residues (30;76).

The antigens presented in MHC class II molecules derive from extracellular proteins that access the endocytic route where they are degraded and become available for binding to MHC class II molecules. However, in normal circumstances, most of the proteins found in the endocytic/lysosomal compartments are not only components of the endocytic route itself but also consist of membrane proteins being internalized and degraded as part of their normal turnover (77). Consequently, most of the peptides eluted from the cell surface MHC class II molecules are endogenous proteins (30). Surprisingly, even in the presence of high concentrations of an exogenous Ag, only a minor fraction of cell surface MHC class II proteins are loaded with peptides derived from that Ag (78-80). However, it is now well established that immature dendritic cells are very efficient in capturing small amounts of Ag (using several pathways) and that upon inflammation-induced maturation, DCs can present these captured Ags at very high levels for a prolonged period of time (10;81-83).

Ag access the endocytic route for presentation by MHC class II through several internalization pathways, these include fluid-phase uptake, pinocytosis, phagocytosis and receptor-mediated endocytosis; the relative contribution of each pathway differing in importance between various APCs (10;84). Receptor-mediated endocytosis is the most specific and can be achieved by different receptors like the B Cell Receptor (BCR), the mannose receptor and the Fc receptors (84). The endocytic route consists of a complex network of compartments that can be divided in three main regions: the early endosomes (slightly acidic pH, low proteolytic activity), the late endosomes (acidic pH and some proteolytic activity) and finally the lysosomes (very acidic pH and rich in proteolytic enzymes) (85). Following internalization, the native structure of the antigen is destabilized by the low pH and disulfide bonds are reduced by an enzyme called Gamma Interferon-inducible Lysosomal Thiol reductase (GILT) (86;87). The cleavage of the reduced antigen in smaller fragments involves an initial cleavage by endopeptidases followed by trimming of the ends by amino- and carboxy-peptidases (88). These endosomal proteases are first synthesized in an inactive form called zymogens which can be activated (by removal of a prodomain) by others proteases or by autoproteolysis (89). Because the activity and the stability of these proteases are pH-dependent, each protease

can be activated at different stages along the endocytic route (77). The lysosomal proteases involved in antigen processing are the cysteine and aspartic proteases called Cathepsins (Cat) (90). The members of the Cat family (17 identified members) show very heterogeneous tissue and cell expression. Their expression and activity can be regulated by various cytokines like IFN-γ, IL-6, IL-10, TNF-α and IL-1β (71;90;91).

Transcriptional regulation of MHC class II is largely mediated by MHC class II *trans*-activator molecule (CIITA), a transcriptional regulator coordinating the function of DNA binding proteins and histone acetylases (92). The class II α and β chains are co-translationnaly inserted into the membrane of the ER where they form heterodimers. The heterodimers interact with a non-polymorphic, highly expressed protein called the invariant chain (Ii), which forms a homotrimer in the ER and functions as a chaperone to favour the proper folding of the MHC class II chains (93-95). Multiples forms of Ii arise from alternative protein transcription initiation sites and alternative splicing; each Ii form is found as a trimer interacting with the MHC class II molecules (84). The stable interaction between the MHC class II and Ii is predominantly mediated by a domain of Ii (spanning residues 81-104) known as class II-associated invariant chain peptide (CLIP) (96). The crystal structure of the complex between MHC class II and CLIP revealed that CLIP binds inside the peptide binding groove of the MHC class II, in a manner similar to the binding of antigenic peptides (75;97). By blocking the peptide binding groove, the invariant chain prevents the binding of endogenous peptides from the ER or the cytoplasm to MHC class II complexes (95;98;99). Properly folded nonameric molecules (three $\alpha\beta$ heterodimers bound to one Ii trimer) are exported from the ER to the Golgi network. The N-terminal (cytoplasmic) portion of the invariant chain contains sorting signals, which then target the MHC class II/Ii complex that reached the trans-Golgi network to endosomal organelles (100-102). In these organelles called MHC class II Compartments (MIIC), the invariant chain is partially degraded at the carboxy-terminus by members of the Cathepsin family, leading to the release of MHC class II heterodimers complexed with the CLIP fragment (101).

The MHC class II/CLIP complexes are then transported to other organelles in which reside the non-classical MHC class II HLA-DM (84;103). HLA-DM has been shown to enhance the release of CLIP from the peptide binding groove and to act as a chaperone to stabilize the intermediate, empty MHC class II molecules (104-108). Moreover, HLA-DM acts as a peptide editor by catalyzing the exchange of low-affinity, unstable peptides for peptides that bind stably the MHC class II (109;110). Experiments using HLA-DM-deficient cell lines and H2-M deficient mice have demonstrated that HLA-DM is required for proper MHC class II/peptide complex formation, antigen presentation and normal CD4 T cell repertoire selection (111;112). In B cells, a non-classical MHC class II molecules called HLA-DO binds to HLA-DM and influences peptide presentation (113-116). The exact function of HLA-DO is still a matter of debate. It is believed that HLA-DO favours the presentation of peptides internalized by the BCR (having a high affinity for the BCR), and, through the specific CD4 T cell-mediated stimulation of these high affinity B lymphocytes, a skewing of the response toward B cells producing high affinity antibodies occurs (115-118). Finally, peptide-loaded MHC class II molecules exit the MIIC and are transported to the cell surface where they can fulfill their function of presenting Ags to CD4 T lymphocytes.

1.1.4 Generation and maintenance of the T cell compartment

Two populations of T lymphocytes, which can be distinguished by the nature of the TCR they express, exist in both mouse and human: the γδ and the αβ T cells. γδ T cells constitute only a small fraction of peripheral blood lymphocytes but represent a major T cell population in the epithelial-rich tissue of the skin, intestines and reproductive tract (119). These cells are characterized by the expression of a restricted set of different TCRs, by the absence of MHC restriction, by the heterogeneity of molecular determinants they recognized (carbohydrates, proteins, alkylamines, superantigens, etc.), by the fact that they are not restricted by presenting MHC alleles and by the heterogeneous biological functions they serve (antimicrobial, tumour surveillance, autoreactive cells elimination, etc.) (119-121). The work described in this thesis focuses on the second subset of T lymphocytes identified as the αβ T lymphocytes. The αβ T cell population

includes two major subtypes having different but complementary functions during immune responses; they are phenotypically distinguished by the expression of the CD4 or the CD8 co-receptor. "Helper" CD4+ T lymphocytes, by the secretion of cytokines and the expression of specific co-stimulatory receptors, are primarily involved in T-dependent B cell responses and in the coordination of the CD8 T cell response (12). "Cytotoxic" CD8+ T lymphocytes (CTLs) directly kill infected or abnormal cells by the polarized secretion of cytokines, lytic proteins and by the expression membrane-bound effector molecules (12).

1.1.4.1 Generating a diverse TCR repertoire: TCR genes and V(D)J recombination

Recognition of MHC/antigen complexes by T cells is achieved by the TCR, a heterodimeric transmembrane molecule formed by the covalent association between an α and a β chain. These monomeric chains are generated by the somatic rearrangement of multiple gene segments within the TCR loci (figure 2); the TCRα and TCRβ loci are located on the long arm of chromosome 14 and on the long arm of chromosome 7, respectively (122;123). During T cell development, distinct regions within each locus are joined to form the complete α and β chains. The TCR β chains are formed by the assembly of different variable (V), diversity (D) and joining (J) regions; the rearrangement of different V and J forms the TCR α chains (no D region within the TCRα locus) (124). These newly rearranged VDJ or VJ gene segments are then joined to the constant (C) region sequences by RNA splicing following transcription (125). The sequencing and analysis of the complete human TCR β locus has revealed that it spans over 685-kilobases of DNA and comprises 65 different Vβ gene segments, 2 Dβ, 13 Jβ and 2 Cβ segments (126). Among the 65 Vβ segments, 46 are functional and 19 represent pseudogenes (not expressed); 1 Vβ segment is found in reversed orientation at the 3' end of the locus. The Vβ segments can be divided into 30 families that share more than 75% nucleotide sequences similarity (127;128). Genes encoding for the TCR α chain comprise 46 functional Vα (8 pseudogenes) and 50 functional Jα (11 pseudogenes) segments followed by a single Cα segment.

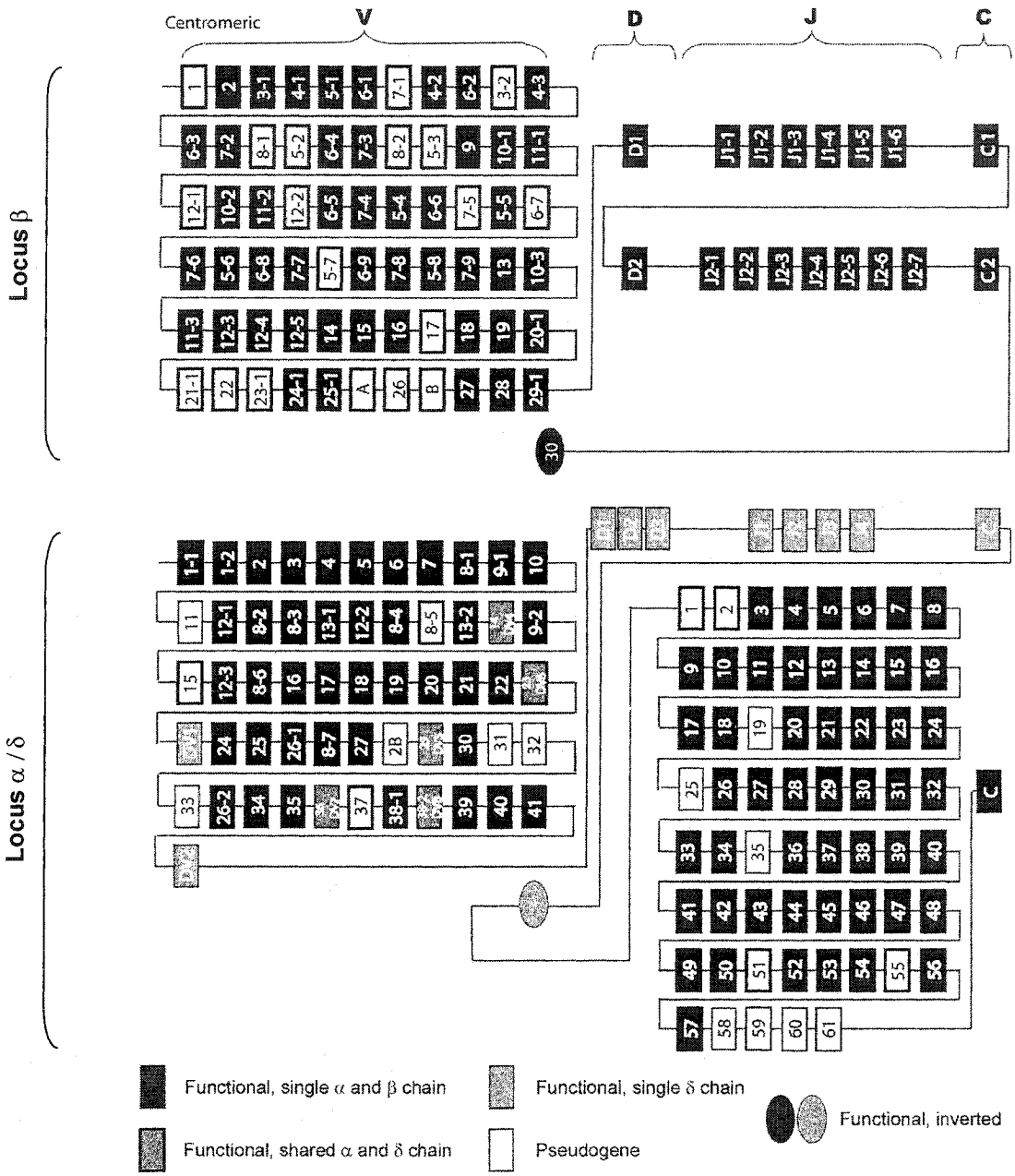

Figure 2. The genomic organization of the human TCR genes. *The gene segments are depicted as classified into V, (D), J and C families and in the sequence in which they are encoded on their respective loci. Based on Glusman et al., Immunity 15(3), 337-339, Copyright (2001), with permission from Elsevier. Genes are named according to the IMGT nomenclature. Correspondence between the different TCR gene nomenclatures can be found at "IMGT, the international ImMunoGeneTics information system® http://imgt.cines.fr (Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France).*

Because the complete sequence encoding the TCR chains is broken up into discontinuous gene segments (V, D and J), a process known as TCR gene rearrangement (or recombination) must take place at the TCR loci in order to join the different segments and generate a functional and diverse set of receptors (analogous to the rearrangement of immunoglobulin light and heavy chain loci in B cells). This process occurs during T cell maturation in the thymus and is a crucial part of early T lymphocyte development; disruption of the recombination process prevents expression of functional TCRs, blocks the generation of mature T lymphocytes and results in severe immunodeficiency.

The different TCR gene segments are flanked by conserved recombination signal sequences (RSS), composed of a conserved palindromic heptamer (consensus: CACAGTG) and an AT-rich nonamer (consensus: ACAAAAACC) separated by non-conserved 12 or 23 base pairs (bp) spacers (129). Recombination only occurs between two gene segments flanked by RSSs that contain 12 and 23 bp spacers, respectively; this is referred to as the 12/23 rule (130). The RSSs are recognized by two proteins called Recombination Activating Genes (RAG)-1 and 2, which are both absolutely required to initiate V(D)J recombination; RAG-deficient mice are characterized by the absence of mature B and T cells and a severe combined immunodeficiency (SCID) (131;132). RAG expression is limited to cells of the B and T lymphocytes lineages and, within these two lineages, high level expression is restricted to the early stages of development during which the pre-immune repertoire of antigen receptors is generated (133). Two waves of RAG expression are observed during normal αβT cell development, corresponding to the successive rearrangement of the TCRβ and α chains (134). RAG contributes to several phases of rearrangment. First, RAG introduces single-strand nicks between two coding sequences and their flanking RSSs. This is then followed by the transient formation of a DNA intra-strand hairpin at the end of each of the coding segments (coding ends) as well as blunt 5' phosphorylated RSS ends (signal ends). These steps are facilitated by the action of two abundant and ubiquitous DNA-binding and bending proteins, the high mobility group (HMG)-1 and 2 (135;136).

The next step involves the opening of the hairpin by the action of an endonuclease; *in vitro* experiments have suggested that the RAG proteins might performed this cleavage step (137;138). The resolution of the hairpin structure intermediates, because of variation in endonuclease cleavage, frequently results in the deletion of a small number of nucleotides and in the addition of short palindromic sequences known as P-regions. (139;140). In addition, untemplated nucleotides may be randomly added *de novo* to joints by the enzyme Terminal deoxynucleotidyl Transferase (TdT), forming what is known as the N-region and thus greatly increasing the potential receptor diversity. Studies using TdT-deficient mice have shown that B and T cell development were normal and no apparent immunodeficiency was observed although the diversity of the peripheral TCR repertoire in these mice was greatly reduced as compared to wild-type mice (10 to 20 times less diverse), mainly due to the complete absence of N-nucleotide regions (141-143). The absence of N-region results in average shorter length TCR β chain complementarity-determining region (CDR) 3, the region encompassing all the rearrangement junctions (143). Random nucleotide loss and addition generate more diversity in the TCR sequence but have the consequence that approximately two-thirds of the potential TCR are eventually assembled out of frame (133).

Processing and joining of the nicked hairpin structures is carried out primarily by ubiquitously expressed nonhomologous DNA end-joining (NHEJ) proteins, which are involved in the repair of DNA double-strand breaks. They include XRCC4, DNA Ligase IV, the three components of the DNA-dependent protein kinase (DNA-PK) consisting of the Ku70 and Ku80 DNA binding subunits and the large catalytic subunit (DNA-PKcs), as well as the newly characterized Artemis (129;139;144). Moreover, there is accumulating evidence that the endonuclease activity leading to hairpin opening is mediated by a complex between Artemis and DNA-PKcs (139;140). Deficiency in any of the NHEJ proteins results in partial or complete block in T and B cell development as well as increased sensitivity to stress causing DNA damage (129). Most notably, DNA-PKcs-deficient mice have been extensively studied and are defined as the classical SCID mice (145-148).

Overall, several mechanisms contribute to the αβ TCR diversity:

- Polymorphism in the gene-encoded V, D and J segments;
- Random recombination of the different gene segments;
- Random deletion and insertion of nucleotides (P- and N-regions) during the rearrangement process;
- Additional diversity is generated by the different pairing between rearranged TCR α and β chains to form the heterodimeric αβ TCR. It was estimated that, in human, a single TCR β chain is paired on average with at least 25 different TCR α chains (149)

1.1.4.2 T cell development

T cells originate from bone marrow-derived hematopoietic stem cells expressing the CD34 marker and having migrated via the blood to the thymus, the primary site of T cell development, through a poorly defined chemotactic mechanism (150;151). The thymus is extremely active during the fetal and perinatal periods; thymic function gradually declines in an age-dependent manner (a phenomenon known as thymic involution) but appears nevertheless to be sustained in older people (152-154). Thymic immigrants enter the thymus in the junction between the cortex and the medulla (155). Following their entry, precursor cells follow a differentiation program characterized by changes in cell-surface marker expression, proliferation status and functionality (150). During the first stages of differentiation, T cell precursors migrate outwards in the cortex before migrating back to the medulla where the final stages of T lymphocyte development take place (155).

Because the lymphoid progenitors entering the thymus have the potential to become T, B or NK cells, the first step in T cell development is the commitment to the T lineage. There is strong evidence that this commitment is driven by engagement of the NOTCH1 receptor by its ligands present at the surface of thymic stromal cells (156-158). The early, committed T cells lack expression of TCR as well as that of CD4 or CD8 co-receptors, and are termed double-negative (DN). As cells progress through the DN differentiation

stage, recombination at the TCRβ locus occurs and a process called β-selection takes place. β-selection refers to the process by which precursor T cells with a productive rearrangement of the TCRβ locus (ie. expressing a functional, in-frame TCRβ chain) receive survival and proliferation signals and are selected to undergo further differentiation (159). In the mouse, it has been shown that β-selection commits precursor cells to the αβ (as opposed to γδ) T cell lineage (160). For β-selection to take place, the cells must express a pre-TCR: the pre-TCR is the result of the pairing of a rearranged TCRβ chain with a pre-TCR-α chain (pTα), which is a protein encoded by a non-rearranging locus (161;162). Expressed at the cell surface, the pre-TCR is associated at the cell surface with a collection of proteins, the CD3/ζ complex, involved in proximal signalling. Active signal transduction through the pre-TCR is required to pass the β-selection checkpoint and for further maturation of the T cell precursors to take place (163;164). Cells emerging from β-selection undergo extensive cell division, lose the expression of pTα, begin to express CD8 and CD4 and undergo recombination at the TCRα locus (165;166). Cells at this stage of differentiation expressing αβ-TCR, CD4 and CD8 are called double-positive (DP) and constitute more than 90% of the lymphoid compartment in the thymus (166).

From the large number of DP thymocytes undergoing a stringent selection process, only a small proportion (less than 5%) that is best suited to function in the host environment will mature and migrate to lymphoid tissues (167). Three different outcomes are possible for the cells submitted to the selection process: death by neglect, negative selection or positive selection (168). The majority (~ 90%) of DP thymocytes express TCRs that interact very weakly with self-peptide/MHC complexes present at the surface of cortical epithelial cells; because the intracellular signals required to sustain cell viability are not generated by this weak interaction, these thymocytes die by neglect (166). A small proportion (~ 5%) of the developing thymocytes possesses TCRs binding strongly to self-peptide/MHC complexes; these T cells could induce autoimmune diseases if they were allowed to leave the thymus. The strong signal delivers to the thymocytes through the TCR induces clonal deletion by apoptosis, a process called negative selection (167).

Finally, cells that bear TCRs that recognize self-ligands and generate signals having an intensity between those resulting from neglect and from negative selection undergo a multi-step maturation process ultimately leading to the differentiation into either CD4+ or CD8+ mature T lymphocytes exiting the thymus and migrating to the periphery (166). Overall, thymic selection constricts the naïve T cell repertoire toward TCRs having a moderate half-life for their cognate pMHC; T cells with the potential to bind foreign Ag with the lowest and highest stability are selectively deleted in the thymus prior to their exportation in the periphery (169)

As outlined above, the selection process promotes the elimination of T cells that could react strongly with self-proteins and induce autoimmunity (a mechanism called central tolerance). One problem is that not all self-proteins are expressed in the thymus, and so T cells carrying TCRs that recognize self-peptides expressed by other tissues would not eliminated. However, it has been shown that RNA transcripts encoding for proteins previously considered to be expressed only in peripheral tissues can be detected in the thymus (170). Recently, mice defective for a transcription factor called autoimmune regulator (AIRE) have been generated; these mice developed several autoimmune disorders (171). It was shown that the AIRE protein, expressed mainly by thymic medullary epithelial cells (MECs), induces the transcription of several genes (evaluated to be between 200 and 1200) encoding peripheral tissue-restricted Ags in these cells (171). Therefore, the presence of AIRE appears to ensure that developing thymocytes are selected against a wide array of self-proteins expressed in peripheral tissues and significantly decreases the probability of exporting self-reactive T lymphocytes in the periphery (172).

For many years, no specific marker identifying recent thymic emigrants (RTEs) was available and the extent of ongoing thymopoiesis was difficult to assess directly. Cell surface markers such as CD45RA, CD27, CCR7 and CD62L are expressed by all naïve T cells so they do not distinguish between RTEs and long-lived naïve T cells (173). Recently, a new molecular marker for RTEs has been identified both in chicken and human (153;174-176). TCR excision circles (TRECs) consist of the intervening gene

fragments that are excised from the genomic DNA and circularized during TCR recombination. Using a quantitative PCR assay, these TRECs can be detected and the frequency of TRECs-containing cells (considered as RTEs) in a given cell population can be accurately quantified. TRECs persist episomally (not integrated in the genomic DNA), they are not replicated and are therefore transmitted to a single daughter cells following mitotic cell division (diluted by T cell proliferation). Therefore, TREC levels not only reflects thymic output but also the extent of proliferation and apoptosis of the naïve T lymphocytes (173;177).

1.1.4.3 αβ TCR structure

TCR structural studies have been hampered by enormous problems encountered in producing large quantities of correctly folded soluble TCRs. In 1995, the crystal structure of the extracellular portion of a murine TCR β chain as well as the structure of the variable portion of a TCR α chain were solved (178;179). One year later, the first TCR αβ heterodimeric crystal structure published was that of the murine 2C TCR (crystallized alone and in complex with its specific peptide/MHC class I, dEV8/H-2K^b) (180). As expected from their amino acid sequence similarities, the overall structure of the αβ heterodimer is very similar to that of the antigen binding region (Fab) of antibodies (181). The Vβ, Cβ and Vα domains of the TCR are related to Ig domains while the Cα domain significantly deviates from the standard Ig fold (180). Comparison of a bound and unbound TCR revealed that, upon binding to the peptide/MHC, conformational changes are induced in the CDR loops (a phenomenon termed "structural accommodations" or "induced fit") (181). The TCR α and β chains are linked by a disulfide bond located in the hinge region adjacent to the transmembrane domain. The hydrophobic transmembrane region (21 or 22 amino acids) anchors each chain in the plasma membrane; the transmembrane helices of both chains are unusual in that they contain conserved basic amino acid residues which enable the αβ chains to interact with chains from the signal-transducing CD3 complex (182). Finally, each TCR chain contains a short cytoplasmic tail (5 to 12 amino acids) that is not involved in signal transduction but,

in the case of the α chain, is necessary for protein kinase C (PKC)-mediated TCR internalization (183).

The crystal structure of more than 10 different TCR/peptide-MHC (class I and II) complexes has been determined so far (180;184-192). Although each of these complexes possesses unique structural features, some general principles ruling TCR recognition of peptide-MHC can be drawn:

- Recognition of the peptide-MHC complex occurs through the so-called "TCR combining site", composed of the variable loops (CDR1, 2, 3 of each chain and the HV4 loop of the β chain) (181). The hypervariable CDR3 loops (having the highest sequence diversity because randomly generated during TCR recombination) from each chain meet and form a pocket over the central amino acids of the peptide. The CDR1α and CDR1β loops are positioned over the N-terminal and C-terminal end of the peptide, respectively. The CDR2 loop of the α chain lies over the MHC class I α2 helix or the MHC class II β1 helix and the CDR2 of the β chain is positioned over the α1 helix (MHC class I and II).
- The TCR interacts with the peptide-MHC complex in a diagonal orientation; the angle between the peptide direction and the long axis of the αβTCR interface is between 45° and 80°.
- The MHC helices dominate the TCR/peptide-MHC interface. The TCR has multiple contacts with highly conserved amino acid residues from each MHC helix, suggesting that TCRs were evolutionarily selected for their ability to recognize conserved features of MHC molecules (193). Most of the TCR contacts with peptides generally involve only one or two amino acids usually located at the center of the peptide.
- Poor shape complementarity between the TCR and the peptide is believed to facilitate the TCR's ability to adapt to different ligands with different biological outcomes, as seen with agonist, partial agonist and antagonist (185). This poor shape complementarity may also explain the relatively short half-life of the TCR/peptide-MHC interaction (181).

- The CDR loops are very flexible (especially those interacting with the peptide) and can adopt different conformations, allowing a given TCR to interact with many structurally diverse peptide-MHC complexes and explaining phenomenon known as cross-reactivity or molecular mimicry (193).
- The crystal structures of the TCR/peptide-MHC complexes are all monomeric and show no evidence of higher order oligomerization.

The TCR does not bind to the peptide-MHC in isolation but as part of a complex with monomorphic co-receptors CD4 and CD8 ($\alpha\alpha$ or $\alpha\beta$ dimer). These co-receptors do not influence the specificity of TCR recognition but they affect the qualitative nature of the binding by augmenting the TCR/pMHC interaction and consequently influencing the biological outcome (194-197).

1.1.4.4 T cell signalling

Signalling in mature T lymphocytes can be divided into three closely interrelated steps. First, TCR signalling begins with an early wave of protein tyrosine kinase activation. This early wave then leads to the activation of multiple downstream signalling pathways. Finally, these pathways activate transcription factors that ultimately lead to the expression of genes that control cellular responses (cytokine/chemokine secretion, proliferation, differentiation, and apoptosis) (198). An overview of T cell signalling pathways is depicted in figure 3.

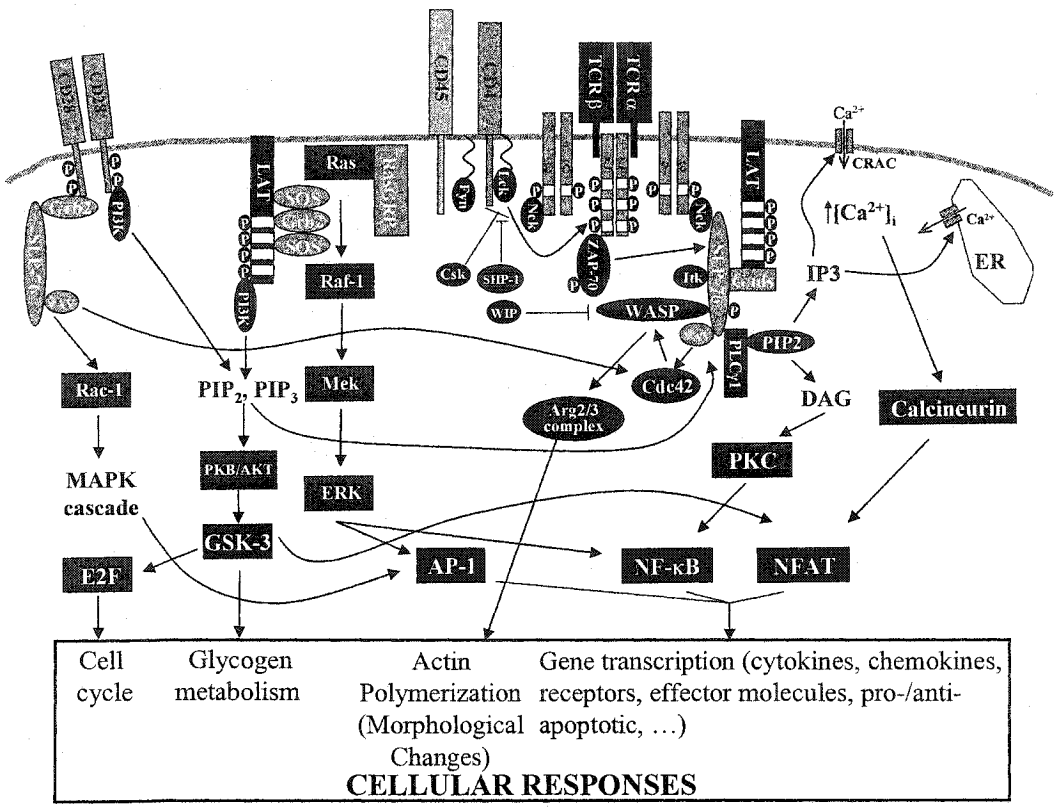

Figure 3. Overview of T cell signalling pathways. *TCR engagement results in the initiation of the intracellular signalling cascade (see text for details). Beyond the direct TCR-induced signals, the engagement of other cell-surface receptors (co-stimulatory receptors, adhesion molecules, cytokine and chemokine receptors) by their appropriate membrane-bound or soluble ligands further modulate T cell responses. The final outcome for the T cell is a broad range of responses that include the up- or down-regulation of surface molecules, the secretion of soluble mediators, proliferation, differentiation and survival/death.*

1.1.4.4.1 The TCR-CD3 complex

As mentioned earlier, the very short cytoplasmic tail of the αβTCR chains are not involved in T cell signalling; they lack any direct enzymatic activity or functionally relevant signalling domains. Therefore, the complete, functional TCR complex is formed by the non-covalent association between the clonotypic αβTCR heterodimer that mediates Ag recognition, and the conserved multimeric CD3 subunit, responsible for signal transduction. The CD3 signalling subunit, which also controls the surface expression of the αβTCR, is composed of four different chains (γ, δ, ε and ζ) assembled as two heterodimers (CD3εδ and CD3εγ) and one homodimer (CD3ζζ) (199). The CD3

chains contains a specific motif [YxxI/L (7-8 amino acids) YxxI/L] called immunoreceptor tyrosine-based activation motif (ITAM) in which both tyrosines serve as substrates for the Src kinases (200). These ITAMs appear as a single copy on the CD3γ, δ and ε chains and as triplicate repeat on the ζ chain, thereby contributing 10 motifs to each TCR complex (201).

1.1.4.4.2 Initial phase of tyrosine protein kinase activation

The first biochemical events occurring upon TCR triggering involve the activation of src-family tyrosine kinases $p56^{Lck}$ (and possibly $p59^{Fyn}$) by the CD45 tyrosine phosphatase (202-204). It has been proposed that this activation results from the transphosphorylation of kinase molecules either in oligomerized (or aggregated) TCRs or in heterodimerized TCR/co-receptor (CD4 or CD8); it is important to note that a fraction of both co-receptors is constitutively associated with $p56^{Lck}$ in resting T cells. It is also hypothesized that tyrosine kinase activation is driven by exclusion of phosphatase molecules away from the TCR-ligand complex (205). The active pool of $p56^{Lck}$ phosphorylates tyrosines residing in ITAMs of the CD3 chains. Interestingly, it was shown that the phosphorylation of the ζ chain ITAMs is accomplished in a sequential, non-random order (206). Phosphorylated ITAMs on the ζ chain constitute binding sites for proteins bearing SH2 domains, such as the cytosolic protein tyrosine kinase (PTK), ζ-associated protein 70 (ZAP-70) (207). After it is attached to the ITAM motifs, ZAP-70 is phosphorylated (activated) by $p56^{Lck}$ (208;209). Active ZAP-70 in turn phosphorylates a central substrate called linker for activation of T cells (LAT), a type III transmembrane protein with a short extracellular region and a long cytoplasmic tail that includes 9 tyrosine-based motifs (210;211). Upon phosphorylation, these motifs serve as docking sites for specific SH2 proteins, phospholipase C (PLC)-γ1, growth factor receptor-bound protein 2 (Grb2), phosphoinositol (PI)-3 kinase and Grb2-related adaptor downstream of Shc (Gads) (201). LAT associates with SH2 domain containing leukocyte protein of 76 kd (SLP-76), leading to activation of PLC-γ1 as well as the Ras pathway. Another adaptor protein called TCR-interacting molecule (TRIM) is believed to be directly phosphorylated by Src

kinases and contributes to downstream signalling by recruiting specific proteins like phosphatidylinositol 3-kinase (PI3K) to the membrane (200;212;213). LAT, TRIM and SLP-76 are called adaptors because they form scaffolds to assemble signal tranduction molecules in the correct intracellular location for them to execute their effector function either directly or after allosteric regulation by co-assembled regulatory proteins (214).

1.1.4.4.3 PLC-γ1 pathway and PKC activation

The PLC-γ1 pathway leads to the generation of second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the release of calcium (Ca^{2+}) from the ER storage sites into the cytoplasm; the rise in cytoplasmic Ca^{2+} $[Ca^{2+}]_i$ in combination with DAG activates an enzyme called protein kinase C (PKC) (215;216). Once the ER stores are depleted, store-operated Ca^{2+} channels in the plasma membrane (CRAC channels) allow extracellular Ca^{2+} influx into the cytoplasm (217). Release from ER stores accounts for the early rise in intracellular Ca^{2+} following TCR ligation, whereas CRAC channel opening is required to sustain the Ca^{2+} signal (200;218). Sustained $[Ca^{2+}]_i$ elevation activates calcineurin, a calcium/calmodulin-dependent serine phosphatase that dephosphorylates members of the Nuclear Factor of Activated T cells (NFAT) (219). Dephosphorylated NFAT molecules translocate into the nucleus in association with transcription factors of the activator protein (AP)-1 family (c-jun and c-fos), bind to NFAT binding sites located in specific gene promoters, such as those regulating IL-2, 3, 4, 13, GM-CSF, TNF-α, FasL and CD40 transcription, and ultimately drive gene transcription (198;219).

In combination with DAG, the elevated $[Ca^{2+}]_i$ activates PKC, an important mediator of signal transduction. T cells express more than 10 functionally distinct PKC isoforms but confocal microscopy experiments have revealed the specific recruitment of PKC-θ at the contact zone between the T cell and the APC (220). Confirming the central role of this enzyme in T cell activation, T cells from PKC-θ-deficient mice fail to proliferate, to secrete IL-2 or to upregulate CD25 (the high affinity subunit of the IL-2 receptor)

following activation by anti-CD3/anti-CD28 antibodies (221). In mature T lymphocytes, PKC-θ couples TCR stimulation to NF-κB induction by phosphorylating IkB Kinase β (IKKβ), which in turn phosphorylates the inhibitory proteins IκBα and IκBβ, targeting them for proteolytic degradation (222). This leads to the release and the nuclear translocation of NF-κB dimers, that then regulate the transcription of genes involved in cellular proliferation and survival (198). PKC-θ is also required for the generation of active AP-1 at a step downstream of the activated Extracellular signal-Regulated Kinase (ERK) and c-Jun N-terminal Kinase (JNK)/ Mitogen-Activated Protein-kinase (MAPK) pathways. Moreover, it was recently shown that PKC-θ deficiency leads to abrogated NFAT transactivation due to reduced IP_3 generation and intracellular Ca^{2+} mobilization (223). By connecting the TCR to the NF-κB, NFAT and AP-1 families of nuclear transactivators, PKC-θ regulates the ability of peripheral T cells to secrete IL-2 and mount normal proliferative responses upon activation (224).

1.1.4.4.4 P21ras pathway

TCR triggering leads to a rapid accumulation of the active, GTP-bound form of p21ras in the vicinity of the T cell membrane (225). Ras activity is controlled by the counter-regulatory effects of guanine nucleotide exchange factors (GEFs), which promote Ras activation, and GTPase-activating proteins, which stimulate the intrinsic GTPase activity of Ras (GTP hydrolysis and Ras inactivation) (225). A well-characterized pathway of Ras activation involves the LAT-associated adaptor Grb2. The SH3 domain of Grb2 recruit a Ras GEF called SOS, the mammalian homologue of the Drosophila "Son of Sevenless", driving its translocation from the cytosol to the plasma membrane where it can activate Ras. Once activated, Ras couples to multiple pathways, including stimulation of the ERK/MAPK cascade as well as Rho GTPases.

1.1.4.4.5 Phosphatidylinositol-3 kinase pathway

PI3Ks are a family of lipid kinases formed by the tight association between a p110 catalytic subunit and a p85 regulatory subunit (226). The p85 subunit contains an SH2 domain, allowing its recruitment to the plasma membrane by tyrosine phosphorylated adaptor molecules (TRIM or LAT); constitutive membrane targeting of p110 creates the active PI3K (227). Active PI3K phosphorylates membrane phosphatidylinositol and its by-products on the D3 position of the inositol ring to generate phosphatidylinositol-3-phosphate, phosphatidylinositol-3,4-bisphosphate (PIP_2) and phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (226). Production of PIP_2 and PIP_3 allows the recruitment to the cell membrane of plextrin homology (PH) domain-containing proteins involved in cell survival or cytoskeleton rearrangement. Proteins whose activity are regulated by PI3K include:

- TEC family tyrosine kinase BTK and ITK. These kinases function at the plasma membrane to regulate PLCγ1 activity.
- Serine/threonine kinase protein kinase B (or Akt/PKB). Akt can phosphorylate the enzyme glycogen synthase kinase (GSK)-3, an important regulator of glycogen metabolism; GSK-3 has also been reported to control the nuclear export of the transcription factor NFATc (228). Also, Akt can stimulate the activity of E2F transcription factors, which are important components of the mechanisms that control the mammalian cell cycle (229;230).
- GEFs for Rho GTPases such as Rac, Rho and Cdc42. The best characterized Rho family GEF in T lymphocytes is Vav-1. PIP_3 induces the recruitment of Vav-1 to the plasma membrane where it is activated by tyrosine kinases (most probably $p59^{fyn}$ and ZAP-70) (231-233). Rho GTPases interact with multiple effectors and can initiate diverse signals, thereby regulating lymphocyte differentiation, proliferation and survival (227). The first biological role ascribed to these GTPases was the dynamic organization of the actin cytoskeleton (227). The activation of Cdc42 by Vav-1 leads to the activation of a protein called Wiscott-Aldrich immunodeficiency syndrome protein (WASP). WASP activates the Actin-Related Protein (Arp)2/3 complex, thereby initiating actin polymerization (233).

1.1.4.4.6 Negative regulation of TCR signalling

In order to limit the extent and duration of TCR activity, especially in the continued presence of ligand, feedback and/or inhibitory mechanisms are also triggered following T cell activation (200). The equilibrium between positive and negative regulators appears to control the outcome of TCR signalling and T cell fate. Several proteins that limit TCR signalling have been described so far:

- C-terminal Src kinase (Csk) is a PTK whose preferred substrates are the inhibitory tyrosine residues of Src-family kinases (234). The binding and enzymatic activity of Csk are enhanced on active (phosphorylated) Lck as compared to inactive Lck, creating a feedback inhibition loop (200;235). Also, Csk activity in T cells appears to be regulated by a protein called Phosphoprotein Associated with GEMs (PAG), although the mechanism by which it does so is not known. (234).
- Although initially identified as a positive regulator of TCR signalling because of its activity on $p56^{Lck}$, studies have shown that CD45 can inhibit or reduce the duration of signalling by inhibiting ZAP-70 and dephosphorylating the CD3ζ chain (236;237).
- Cbl family proteins contain a ring finger domain that interacts with E2 ubiquitin–conjugating enzyme. C-Cbl and Cbl-b are two members of the Cbl family; they are expressed in cells from the hematopoietic lineage (238). T cells from c-Cbl/Cbl-b double knockout mice show impaired ligand-induced TCR internalization, leading to sustained TCR signalling and hyperresponsiveness following TCR stimulation (238). These results suggest that Cbl family proteins negatively regulate T cell activation by promoting down-modulation of TCRs from the cell surface, a mechanism that seems important for the termination of TCR signals.
- SH2 domain-containing tyrosine phosphatase-1 (SHP-1) is expressed in cells from the hematopoietic lineage and is a negative regulator of immunoreceptor signalling (234). SHP-1 can bind the intracytoplasmic region of various receptors with inhibitory activity having a specific motif (I/V/L/S)xYxx(L/V)

called immunoreceptor tyrosine-based inhibitory motif (ITIM). It has been reported that SHP-1 can dephosphorylate multiple proteins involved in signalling such as ZAP-70, Src family kinases, SLP-76, Vav and PI3K (234). Moreover, SHP-1 has been shown to be present in the TCR complex (200;239;240).

- PEP, a protein from the PEP family of nonreceptor protein tyrosine phosphatase (PTP), is expressed both in thymocytes and in mature T lymphocytes. Overexpression of PEP in T cells leads to an inhibition of TCR signalling. It is constitutively associated with Csk, an association that seems to be required for their inhibitory activity; the PEP-Csk complex mediates its action by dephosphorylating the positive regulatory site of Lck and ZAP-70 (241). Another protein from the same family, PTP-PEST, constitutively interacts with several signalling molecules and inhibits the Ras/MAPK signalling pathway (242).

1.1.4.4.7 Contribution of co-stimulatory receptors to T cell signalling

The central event controlling T cell activation is the antigen-specific interaction between TCRs and pMHC complexes presented by the APC. Simultaneously, other accessory molecules present at the T cell surface interact with their specific ligand on the APC. As described in the previous sections, TCR ligation triggers several signalling cascades inside the T cell, leading to specific gene transcription and ultimately controlling T cell activation, differentiation and death. However, because a large fraction of the accessory receptors are also linked to intracellular signalling pathways, they can complement or modify the signals provided by the TCR, thus significantly influencing T cell fate. Here is a description of co-stimulatory molecules involved in T cell activation.

The CD28 receptor, a 44 kD member of the immunoglobulin (Ig) superfamily, was the first molecule shown to function as a co-stimulatory receptor. It is expressed constitutively as a disulfide-linked homodimer on a large fraction of T cells (CD4 and CD8) (243). CD28 interaction with its two ligands on the APC, B7-1 (CD80) and B7-2 (CD86), has been shown to influence T cell response in a variety of ways: driving T cell

proliferation and cytokine production, prevention of anergy and upregulation of anti-apoptotic molecules (243). Cross-linking of CD28 induces tyrosine phosphorylation of the cytoplasmic tail, allowing interaction with signalling adaptors/mediators such as Gads, Grb2 and PI3K. Grb2 recruits SLP-76 and Vav, which in turn activates Rac and Cdc42 leading to cytoskeleton rearrangement via WASP and to IL-2 gene transcription via the MAPK cascade; PI3K can signal by recruiting Akt, leading to NF-κB nuclear translocation and gene transcription (IL-2, Bcl-xL) (244;245).

Cytotoxic T lymphocyte antigen 4 (CTLA-4) was the second member of the CD28 family to be identified and is probably the best-studied inhibitory receptor. Like CD28, it binds to CD80 and CD86 but with higher affinity. It was recently demonstrated that CD80 (but not CD86) strongly favours engagement of CTLA-4 over that of CD28, consistent with their similar pattern of expression; both CD80 and CTLA-4 are not expressed on resting cells, but are upregulated following APC and T cell activation (246). CTLA-4 is found at the cell surface as a disulfide-linked homodimer that can binds bivalently to B7-1 and B7-2 (247;248). The central role of CTLA-4 in negatively regulating T cell activation and peripheral tolerance is illustrated by the fact that CTLA-4-deficient mice die from massive lymphoproliferative disorders and fatal multiorgan tissue destruction within 3 to 4 weeks (249-251). CTLA-4 interferes with T cell activation by two mechanisms. First, CTLA-4 has higher affinity for B7, so it outcompetes CD28 for B7 binding and therefore can abrogate the co-stimulatory signal provided to the T cell by CD28 ligation. Second, CTLA-4 also engages inhibitory signal transduction machinery, including the phosphatases SHP-2 and PP2A, which may neutralize the action of kinases upstream of CD3 and CD28 (244). CTLA-4 triggering has been shown to inhibit activation of ERK and JNK and to reduce phosphorylation of LAT and TCR ζ chains (252;253).

Inducible co-stimulator (ICOS) is a co-stimulatory receptor homologous to CD28, expressed at very low levels on naïve T cells but upregulated upon stimulation (254). The ligand for ICOS, ICOS-L, is expressed on unstimulated B cells, macrophages and dendritic cells (255). Engagement of ICOS on TCR-stimulated cells results in increased proliferation and cytokine secretion (254). ICOS-deficient mice have impaired humoral

immunity, highlighting the role of the ICOS pathway in B cell help and germinal center formation (256-258). These defects are due to a failure to upregulate CD40L on CD4 T cells, impairing T cell help (254). The signalling cascade coupled to ICOS is largely unknown; the cytoplasmic tail of ICOS contains a motif that binds the p85 subunit of PI3K and is associated with lipid kinase activity (259).

Program death-1 (PD-1) is a member of the Ig superfamily expressed on T cells following activation through the antigen receptor (255). The ligands for PD-1 are two members of the B7 family called PD-L1 (B7-H1) and PD-L2 (B7-DC); they are not expressed by resting professional APCs but transcripts for these ligands can be detected following activation (255). Engagement of PD-1 by its ligands results in T cell response attenuation as measured by decreased proliferation and cytokine production (260;261). PD-1 cross-linking results in rapid tyrosine phosphorylation of SHP-2, a phosphatase involved in negative signalling (262). PD-1 deficiency results in autoimmune disorders, suggesting that this receptor has a critical role in the maintenance of peripheral tolerance (255).

CD2, a member of the Ig superfamily, is highly expressed on all T cells and binds CD58 (LFA-3) expressed on APCs. The cytoplasmic tail of CD2 contains 5 proline-rich stretches that are responsible for the direct physical interaction of CD2 with various intracellular proteins such as CD2AP, CD2BP1 and CD2BP2. CD2AP is coupled to cell polarization and cytoskeletal rearrangement by WASP while CD2BP1 and CD2BP2 have been implicated in adhesion and signal transduction, respectively (263;264). There is evidence that $p59^{Fyn}$ interacts with CD2 and thereby connects CD2 signalling with the MAPK pathway, the activation of PLCγ1 and the adaptor proteins Vav and LAT (265). T cells from CD2-deficient mice are 10 times less sensitive to Ag compared to their wild-type counterparts, both *in vivo* and *in vitro* (266).

Beyond the induction of T cell signalling pathways by direct interaction with cell-surface receptors from the APC, the cellular response can be further directed by soluble factors such as cytokines and chemokines present in the immediate environment. T cells possess receptors couples to signalling cascades recognizing a variety of these soluble mediators.

The majority of cytokine receptors are coupled to the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway as well as to the MAPK pathway (267). Cytokine signal transduction is inhibited by a family of intracellular proteins called suppressors of cytokine signalling (SOCS), which target essential signalling components for proteosomal degradation (268). Chemokine receptors are G protein-coupled receptors, which consist of an extracellular NH_2-terminus, seven transmembrane domains and a cytoplasmic COOH-terminus coupled to heterotrimeric G_i proteins. The chemokines are important regulators of leukocyte trafficking (chemotaxis) but recent studies have demonstrated that they also play a role in T cell differentiation (269). Chemokine receptor engagement triggers several effectors such as PLCβ2 and 3, PI3K as well as MAPK (270).

1.1.4.5 Ligand discrimination by the TCR: sensing the environment and relaying the information inside the T cell

1.1.4.5.1 General considerations

The TCR:pMHC interaction differs strikingly from that of other receptor:ligand systems (such as hormones, growth factors, cytokines). These latter, "typical" receptor:ligand pairs are formed by gene-encoded, non-polymorphic proteins that coevolve; therefore, the structure and the affinity of the interaction, as well as the mechanism of activation (dimerization, conformational change, etc), are defined and predictable. Such predictability ensures the specificity of the interaction, a given ligand being able to trigger only its specific receptor. In contrast, the structure of the TCR, and particularly its ligand-binding region, is highly variable and unpredictable, mainly because of the previously discussed random addition/deletion of nucleotides in the CDR3 region during TCR recombination. Furthermore, the structure of the ligand (pMHC) is also diverse and unpredictable because of MHC polymorphism and due to the high variation in epitopic peptide side chains that may be loaded in the MHC's binding groove. Adding to the exquisite complexity of TCR:pMHC interaction, the TCR must often detect few specific Ag/MHC complexes among thousands of non-antigenic, yet structurally related, self-

peptide/MHC complexes that are present at the surface of the APC. Therefore, the mechanism for regulation of TCR signalling must combine flexibility, to accommodate the highly diverse set of TCR:ligand complexes topologies, and sensitivity, to discriminate between structurally similar ligands that sometimes differ by a single amino acid (271).

TCR:pMHC interactions are of low affinity (K_D = 0.1-500 μM) and are characterized by slow association and fast dissociation reactions (272;273). The relationship between these kinetic parameters for protein-protein interaction is illustrated in the following formulas:

$$\mathbf{K_D} = \frac{k_{off}}{k_{on}} \quad \text{where} \quad \begin{array}{l} K_D = \text{affinity constant} \\ k_{on} = \text{association rate constant (on-rate)} \\ k_{off} = \text{dissociation rate constant (off-rate)} \end{array}$$

$$\textbf{Half-life } (t_{1/2}) = \frac{1}{k_{off}}$$

TCR:pMHC complexes usually exhibit fast dissociation rates corresponding to half-lives of 0.1 to 70 seconds (s) (274). Generally, half-lives have been found to correlate with the functional potency of the T cells bearing the TCR (185;206;275-281), although there are exceptions (206;282-286). The major limitations of these studies is that most of the binding analyses involves soluble TCRs and that it is not known whether removal of the TCRs from their membrane environment affects their ligand-binding properties. Also, the elimination of the transmembrane and cytoplasmic segments, as well as the absence of CD3 chains, may alter the relative orientation or stability of the remaining domains and affect kinetic parameters.

In an elegant study, Mark Davis and colleagues demonstrated that the binding of the TCR to its cognate pMHC complex occurs in two steps (287). First, the more rigid CDR1 and CDR2 loops of the TCR scan the MHC scaffolding and form specific contacts with residues from the MHC α helices, orienting the TCR over the peptide (transition state). In the second step, the structurally flexible CDR3 loops form strong specific peptide

contacts by induced-fit binding (283;287-289). The first step primarily determines the on-rate of the interaction while the second step mostly dictates the half-life of the TCR-pMHC complex. This two-step mechanism would explain how T cells can scan thousands of peptides displayed by MHC molecules at the surface of APCs. It also explains the inherent ability of the TCR to cross-react with structurally different peptides (271;290), because the flexible CDR3 loop can adopt different conformations to make contacts with the Ag (291).

1.1.4.5.2 Models for T cell activation

Several models based on experimental data have been proposed to explain how ligand discrimination and initiation of signalling could be achieved by the TCR complex.

1. **Ligand-induced conformational change model**. According to this model, TCR engagement by its cognate pMHC induces a conformational change in the TCR/CD3 complex that can trigger the signalling machinery; a good example of this mechanism is provided by G-protein-coupled receptors (292). Recent crystallographic data comparing bound versus free TCR structure (the LC13 TCR, recognizing an EBV peptide complexed to HLA-B8) revealed a conformational change in a small loop located in the TCR α constant domain, which has been proposed to be an ideal location for an induced fit-type interaction with the CD3 subunits (293-296). Moreover, it was recently shown that engagement of the TCR complex induces a conformational change resulting in the exposure of a proline-rich sequence in the CD3ε chain, leading to recruitment of an adaptor protein called Nck (297). The Nck family of adaptor proteins are thought to be implicated in organization of actin cytoskeleton and cell movement (298).

Although these data support the notion that a conformational change of the TCR complex occurs, it is still not clear how ligation at the TCR binding site is communicated to the small loop that is a great distance away from the TCR-

pMHC interface. Also, such ligand-induced conformational change has not been observed in numerous other TCR:pMHC complexes and could simply reflect an inherent flexibility of that region. Moreover, the fact that there are no major conformational differences between crystal structures of a TCR bound to four pMHC complexes (same MHC, different peptides) despite the drastic differences in the signalling outcomes following TCR engagement by these complexes argue against the conformational change model (185). It must be noted that crystallographic data of engineered TCRs are collected under conditions which considerably differ from those prevailing in the natural environment of a T cell. It is possible that subtle conformational changes, which cannot be detected in crystallography studies, occur in the transmembrane and/or cytoplasmic domains of the natural, membrane-bound TCR.

2. **Ligand-induced dimerization/oligomerization model.** This model is largely based on the activation of membrane receptor tyrosine kinases. Binding of the ligand induces the dimerization/oligomerization of these receptors, resulting in activation of their cytoplasmic tyrosine kinase domains and trans-phosphorylation of their tails (299). Initial support for ligand-induced TCR oligomerization came from the observation of a "dimer of dimers" in the crystal structure of the MHC class II HLA-DR1 (74). The first direct evidence of ligand-induced TCR oligomerization came from experiments by the group of Mark Davis. Using a technique called Quasi-Elastic Light Scattering (QELS), they have shown that mixing soluble TCRs with their cognate pMHCs at high concentration in solution leads to high-order assemblies of up to six TCR-ligand complexes (300). Interestingly, this oligomerization was exclusively observed when the TCRs were mixed with MHCs complexed with agonist peptides. Using the surface plasmon resonance technology (BIAcore^{TM}), another group has demonstrated that, at 37°C, the kinetics of TCR binding to an agonist ligand deviate from simple 1:1 binding and were more consistent with a TCR dimerization model (276). Furthermore, the requirement of TCR

dimerization/oligomerization for T cell activation has also been suggested by indirect functional experiments. Using various TCR systems, several groups have independently shown that soluble dimeric or oligomeric ligands are much more efficient T cell stimulators than monomers (301-304).

Nevertheless, the concept of TCR dimerization as a general model for T cell activation has been challenged by recent experimental observations. Using the same techniques as the two previously mentioned groups (QELS and BIAcore^{TM}) and complementing their study by sedimentation equilibrium analytical ultracentrifugation experiments, Baker *et al.* did not observed any evidence for TCR oligomerization in two separate human αβTCR:pMHC complex systems (305). Moreover, no evidence of dimerization was detected in the crystal structure of several TCR:pMHC complexes (181). Recently, using a refined live-cell imaging method, Mark Davis' group presented convincing data that a single ligand at the surface of an APC can trigger a measurable intracellular response, strongly suggesting that TCR oligomerization is not an absolute requirement for T cell activation (306;307). These data were in agreement with previously published results (308;309). On the basis of these results, combined with structural data suggesting that co-engagement of one CD4 molecule with both the TCR and MHC portions of a single receptor-ligand complex is highly unlikely, a new "pseudodimer" model has been proposed (306;307;310). This pseudodimer would consist of a foreign pMHC-engaged TCR cross-linked by one CD4 molecule to a different, self-pMHC binding TCR. Even though this hypothesis is far from proven, it would explain why oligomeric ligands are more effective T cell stimulators and how only few antigenic peptides at the surface of an APC loaded with thousands of self-peptide can trigger a T cell response. Consistent with the hypothesis that self-pMHC could contribute to TCR signalling, it has been shown that there is an accumulation of both foreign and self-pMHC molecules at the T cell-APC interface (311). It is also possible that no specific TCR oligomerization is taking place but rather that this could be the result of non-specific aggregation

of TCR molecules into the so-called supramolecular activation clusters (SMACs) following T cell activation (this subject will be discussed in details later) (312).

3. **Kinetic proofreading/discrimination model.** This model is largely based on the extensive work on altered peptide ligands (APLs). APLs consist of peptide analogs generated by introducing single amino acids substitutions at residues making contact with the TCR (313). These analogs, along with the native peptide, are tested for their capacity to stimulate a variety of T cell responses. By comparing the T cell responses they trigger with those from the native peptide, APLs can be classified in different categories (table I) (314;315).

Ligand	Biological response
Strong agonist	Full T cell activation; cytolysis, proliferation, cytokine secretion, differentiation
Weak agonist	Same as strong agonist, but requires more ligand for same degree of activation; slower activation kinetics
Partial agonist	Induces some, but not all, biological effects of agonist (e.g. cytolysis but not proliferation and cytokine secretion)
Antagonist	Does not induce activation on its own and inhibits activation by agonist
Null	No detectable activation and biological effect

Table I. Definition of altered peptide ligands.

Because they only trigger select cellular functions, much of the focus has been placed on partial agonists in order to understand the parameters governing T cell activation. T lymphocytes stimulated by a partial agonist show an altered

pattern of tyrosine phosphorylation similar to the pattern observed for T cells interacting with APCs bearing MHCs loaded with self-peptides; the most striking feature of which being a predominance of the hypophosphorylated (p21) form (over the fully phosphorylated p23 form) of the CD3ζ chain (316-318). This is believed to be caused by the inefficient recruitment and/or activation of Src-family kinases; the incomplete phosphorylation of the CD3ζ chain resulting in reduced recruitment and activation of ZAP-70 (313;316;319). An important concept that emerges from experiments with APLs is the fact that the TCR does not seem to work like an on/off switch but more like a rheostat. According to the kinetic proofreading/discrimination model, a certain amount of time is required to complete the stepwise phosphorylation and recruitment of downstream signalling molecules by the ITAMs of the CD3 chains (320;321). The half-life of the TCR-ligand interaction must be long enough to allow the completion of these biochemical reactions and interruption of the interaction due to a rapid dissociation rate leads to an incomplete signal.

Characterization of the kinetic parameters of different TCR:pMHC interactions is in agreement with this model. The affinity of antagonist and partial agonist peptides for TCR is much lower than that of agonists, mostly due to a faster dissociation rate (276;277;281;282;322). Furthermore, this model concords well with the two-step binding mechanism of the TCR proposed by Mark Davis and colleagues (287). According to this mechanism, the critical step leading to T cell activation is the stabilization of the complex by the contacts between the CDR3 loop and the peptide, that, in turn, primarily dictates the dissociation rate of the TCR-pMHC interaction. Therefore, the stabilization of the transition state complex by an agonist peptide that cannot be achieved by an APL or a self-peptide would permit ligand discrimination and complete signalling inside the T cell. Failure of APLs and self-peptides to stabilize the transition state complex would lead to premature dissociation and incomplete signalling. Finally, it is interesting to note that the biological activity of other receptors, such as the

high-affinity IgE receptor (FcεRI) and the BCR, appears to be under the constraints of kinetic proofreading (323-325).

4. **Serial triggering model**. As mentioned earlier, foreign pMHC complexes are usually rare at the surface of an APC and they are present amongst a majority of MHCs loaded with self-peptide. How such a low number of ligands can trigger a strong T cell response has always puzzled immunologists. Using TCR internalization as a direct measure of TCR engagement, the group of Antonio Lanzavecchia observed that the loss of TCR from the cell surface greatly exceeds the number of ligands presented by the APCs (up to a 200:1 ratio) (326). Making the assumption that only engaged TCRs are internalized, they proposed that one ligand can serially trigger several TCRs, generating an amplification mechanism to ensure T cell response even in the case of very low ligand levels (326;327). T cells accumulate short-lived intracellular signalling intermediates originating from serially triggered TCRs, up to a threshold level required for activation of a cellular response (temporal summation) (328;329). From their results, Viola *et al.* deduced that T cells "count" the number of engaged TCRs and respond when a given threshold is reached (8000 and 1500 TCRs in absence or presence of co-stimulatory signals, respectively). According to this model, the half-life of the TCR-ligand interaction must be long enough to allow complete signalling (as stipulated by the kinetic proofreading model) but not too long to permit the serial triggering of multiples TCRs (326). In agreement with this assumption, it was shown, using hybridomas expressing TCR variants, that either decreasing or increasing the half-life of the TCR-pMHC interaction is detrimental for T cell activation; efficient T cell activation occurs within an optimal dwell-time range (285).

However, using a similar system of hybridomas transfected with engineered TCR mutants, Kranz and colleagues did not obtained similar results. They demonstrated that TCR variants having a very high affinity for pMHC complexes (with much longer half-life) show increased TCR down-modulation

and are activated at much lower peptide concentrations as compared to wild-type TCRs (278;330;331). Experiments studying the mechanism of TCR internalization have also raised some doubts about the validity of the serial triggering model as initially presented by Viola *et al.* In an elegant study, Vignali and colleagues demonstrated that activation-mediated TCR down-modulation is caused by the failure to re-express TCRs (because they are retained inside the cell or degraded) rather than by ligand-induced internalization (332). It was also reported that TCR triggering with either antibodies, antigen or superantigen leads to the downmodulation of both engaged and nonengaged TCRs (333;334). These results strongly suggest that the number of internalized TCRs cannot be assumed to equal the number of triggered receptors, which was the main assumption supporting the serial engagement model (326;334). Moreover, the fact that T cells bearing as few as 500-1000 TCRs at their surface can respond to antigenic stimulation is inconsistent with the notion that T cells must serially trigger at least 1500 TCRs to reach their activation threshold (335-337). Therefore, even though the phenomenon of serial engagement most likely occurs during T cell stimulation (338), the notion that T cells "count" the number of internalized TCRs and respond to a given threshold of internalization does not correlate with new experimental data.

1.1.4.6 The dynamic nature of the T cell membrane: lipid rafts and the immunological synapse

There is now accumulating evidence that the plasma membrane is not a uniform lipid bilayers; it contains multiple detergent-resistant, sphingolipid- and cholesterol-rich microdomains called lipid rafts (339). They are evolutionarily conserved structures and they have been shown to be implicated in the signalling process of a variety of receptors (339). Their important role in cell function is related to their ability to specifically segregate proteins in the membrane, thus allowing for the selective local enrichment of

certain proteins in the lipid rafts and the effective exclusion of others from these rafts. The majority of transmembrane proteins are not constitutively located in rafts, but some of them become raft-associated upon cell activation. A small number of transmembrane proteins predominantly localize in lipid rafts, targeted there because they are palmitoylated at specific cysteine residues. Importantly, CD4 and CD8 co-receptors, as well as LAT, have been shown to be raft-resident molecules in T lymphocytes (340-342). In resting T cells, rafts are also enriched in proteins involved in T cell signalling, including $p56^{Lck}$, Cbl, Csk, Ras, PI3K and Grb2. Following T cell activation, several other molecules are recruited into lipids rafts including the TCR complex, ZAP-70, Vav, SLP-76, PLC-γ1, WASP and PKC (339). Interestingly, TCR/CD28 co-engagement leads to an increase in the concentration of lipid rafts on the cell surface and to their redistribution to the T cell/APC interface (343-345). Therefore, rafts appear to play an important role in the spatial organization of the TCR signalling machinery, as illustrated by experiments showing defective T cell activation when key signalling molecules ($p56^{Lck}$, LAT) are mutated to abolish their recruitment into lipid rafts (339).

During the last five years, the development of high-resolution, real-time microscopy has allowed immunologists to gain insight into the earliest events occurring following T cell contact with an APC. Using three-dimensional immunofluorescence, Monks and colleagues published one of the first studies about the reorganization and the segregation of adhesion molecules and TCR-associated components at the T cell/APC interface (312). They showed that upon T cell/APC interaction, these molecules segregate into two major compartments (areas), referred to as supramolecular activation clusters (SMACs). The "central" SMAC (cSMAC) is enriched in TCRs, CD2, CD28, $p56^{Lck}$ and PKC-θ; the "peripheral" SMAC (pSMAC) contains the adhesion molecule Lymphocyte Function Associated (LFA)-1 as well as talin (312;346). This specific segregation and spatial organization of molecules involved in T cell activation in the zone of cell-cell contact is now referred to as the immunological synapse, an analogy with the adhesive junction between neurons (347-349). A synapse (derived from Greek, meaning "connection") can be defined as "a stable adhesive junction between two cells across which information is relayed by directed secretion" (349).

The first dynamic pictures of the multimolecular choreography taking place during formation of the immunological synapse were provided in 1999 by the group of Michael Dustin (348). In order to mimic the membrane of an APC, they incorporated fluorescently-labelled, glycosyl-phosphatidylinositol (GPI)-linked pMHC and ICAM-1 molecules into lipid bilayers on glass supports. Observing the movement of these fluorescent molecules on the planar lipid bilayers by real-time video imaging allowed them to deduce the movement of their respective ligand (TCR and LFA-1) on the CD4 T cell. In the first thirty seconds after contact with the lipid bilayers, T cells stop migrating and develop contact points in which ICAM-1 molecules are segregated into a central region surrounded by an outer ring of pMHC complexes. This initial molecular configuration, called the "immature" immunological synapse, is accompanied by an increase in intracellular Ca^{2+}, demonstrating that this peripheral TCR engagement is functional and leads to the activation of T cell signal transduction pathways. During the next five minutes, the pMHC complexes, by an actin-dependent mechanism, move toward the center of the contact area and form a cluster while the ICAM-1 molecules move away from the center and form a peripheral ring (bull's eye pattern). This final molecular configuration called the "mature" immunological synapse is very similar to the previously described SMACs and appears to be stable for more than an hour, probably the time required to sustain secondary messenger generation and for T cell commitment. They also demonstrate that full T cell activation occurs only if a minimal density of pMHCs is accumulated in the central cluster of the mature immunological synapse. Another important finding was the establishment of a direct correlation between the half-life of the TCR-pMHC interaction and the density of pMHC complexes accumulated in the central cluster, in agreement with the kinetic proofreading/discrimination model. Largely based on these results, it was hypothesized that the role of immunological synapse is to facilitate sustained TCR signalling by concentrating both TCRs and pMHCs, as well as co-receptor/co-stimulatory molecules and theirs ligands, in the center of the contact area (350).

This original *in vitro* model has provided valuable information concerning the movements and the distributions of various proteins in the developing immunological synapse. Since the publication of this paper, several groups have been studying the synapse in more physiological conditions and the model for immunological synapse regulation originally proposed by Grakoui *et al.* has been refined.

Using splenocytes as APCs, Lee *et al.* showed that TCR triggering induces signalling well before the mature synapse is formed, implying that TCR signalling does not require formation of the immunological synapse (351). Furthermore, they clearly demonstrated that TCR-mediated tyrosine kinase signalling, as measured by the presence of phosphorylated $p56^{Lck}$ and ZAP-70, is significantly decreased before mature synapse formation, strongly suggesting that the consolidation of the synapse does not sustain or enhance signalling. These observations raise the question: what is the role of the immunological synapse? Some argue that it is required for the polarized secretion of soluble factors (352). This view is supported by experiments on CD8 T cells showing that membrane "bridges" are formed between the T cell and the target cell, with lytic granule secretion occurring within the ring formed by adhesion molecules in the synapse (353;354). A recent report suggests that TCR signalling and the immunological synapse could function in a cooperative manner to ensure full commitment to T cell activation (355). While continuous TCR signalling is necessary for immunological synapse formation and maintenance, the synapse creates an optimal microenvironment for continuous TCR:pMHC interaction.

Because of its association with $p56^{Lck}$ and with MHC class II molecules, the CD4 co-receptor plays an important role during T cell activation; it increases T cell sensitivity to Ag by 10- to 100-fold (194;197). To better understand the role played by CD4 and $p56^{Lck}$ during T cell activation, their localization during immunological synapse formation has been followed and compared to the movements of the CD3ζ chain (356;357). Few minutes after T cell/APC contact, CD3ζ, CD4 and $p56^{Lck}$ co-localize in a central cluster in the contact area. However, while CD3ζ is stabilized in this central cluster, CD4 and $p56^{Lck}$ rapidly move toward the periphery. Therefore, CD4 does not appear to stabilize

the TCR-pMHC complexes but rather to be involved in the initial phase of activation, possibly by bringing $p56^{Lck}$ into proximity with the CD3 chains to initiate tyrosine phosphorylation (357). Another study showed that the presence of an antagonist peptide inhibits the intermolecular interaction between CD4 and TCR at the T cell-APC interface, an interaction that normally occurs following recognition of an agonist ligand (358). However, this inhibition does not decrease the recruitment and the partial co-localization of CD3ζ or CD4 at the immunological synapse.

To assess their role in the regulation of T cell activation, the localization and the function of CD28 and CTLA-4 in the immunological synapse have been studied. CD28 is localized in the cSMAC and its co-engagement with the TCR leads to increased lipid raft aggregation as well as to sustained activation of $p56^{Lck}$ and Vav (343). In turn, Vav activation leads to the activation of WASP, that induces cytoskeleton rearrangement through actin polymerization, a process critical for membrane reorganization and synapse formation (359). According to this hypothesis, CD28 engagement would synergize with the TCR to amplify the initial signals and promote immunological synapse formation. However, it was demonstrated in another study that the CD28/CD80 interaction appears to be dispensable for the initial step of synapse formation in naïve T cells; the immunological synapse would rather create an optimal cellular microenvironment that favours co-stimulatory molecule interaction and ultimately promotes signal amplification (360). CTLA-4, which is only expressed following T cell activation, accumulates at the T cell/APC interface upon stimulation with an agonist peptide (361). Interestingly, the extent of CTLA-4 accumulation at the synapse is dependent on TCR signal strength; stimulation with an agonist peptide promotes very effective CTLA-4 translocation as compared to weak agonist. These results demonstrate that the composition of the immunological synapse can be modulated by TCR signal strength, thereby providing a means by which to selectively activate and/or sustain specific T cell signalling pathways (361).

1.1.5 Anatomy of T cell-mediated immune response

All of the cellular processes described in the previous sections occur in a highly regulated manner during a normal immune response. From antigen encounter to antigen clearance, several specialized cell types exert their functions in specific anatomical sites. In order to coordinate and synchronize their action, cells involved in the immune response "communicate" with each other either by direct cell-cell contact or via secreted soluble mediators such as cytokines and chemokines.

As mentioned earlier, the αβ T cell compartment can be divided into two major functional subsets; the cytokine-secreting CD4 "helper" T cells and the "cytotoxic" killer CD8 T cells. While cytokine secretion by CD4 T cells controls the function of different cell types including CD8 T cells, B cells and macrophages, the major role of CTLs is to directly kill infected cells by cytotoxic mechanisms.

1.1.5.1 CD4 T cell activation during the primary immune response

One major obstacle to the study of CD4 T cell responses has been that naïve CD4 T cells are primarily found within the T cell-rich areas of secondary lymphoid organs (lymph nodes, spleen and Payer's patches) (362). This preferential localization is explained by the fact that they express a unique set of receptors which bind ligands specifically expressed on the blood vessels of these lymphoid organs. Naïve T cells express CD62L, a member of the selectin family of adhesion molecules which mediates, upon binding to its ligands (Glycam-1 and CD34), the initial attachment and rolling of leukocytes to venular endothelial cells (363). They also express CC chemokine receptor 7 (CCR7), which binds to its ligands EBI1 Ligand Chemokine (ECL or CCL19) and Secondary Lymphoid-tissue Chemokine (SLC or CCL21) presented by mucin-type molecules on the high endothelial venules (HEV) and triggers integrin activation that in turn leads to the arrest of the rolling lymphocyte and its migration across the endothelium (364). The striking paucity of lymph node T cells in CCR7-deficient mice highlighted the importance of CCR7 for T lymphocyte homing to the T cell zone of secondary lymphoid organs (365). Naïve CD4 T cells do not permanently reside in a given lymphoid organ; they continuously recirculate between different lymphoid tissues using the blood flow (362).

Dendritic cells appear to be the only APCs capable of stimulating naïve CD4 T cells and of initiating *de novo* T cell responses *in vivo* (9). DCs are present in most tissues in a so-called "immature" state; they are unable to stimulate T cells because they do not express important co-stimulatory molecules such as CD40 and CD86. However, these DCs possess many antigen-capturing Fc receptors, making them good sentinels to detect and capture foreign Ags present in peripheral tissues. Antigen uptake provides a signal for the DCs to undergo phenotypic and functional maturation, decreasing their ability to capture antigens but increasing their antigen processing capacity. The captured Ags are then processed and presented as peptides bound to MHC class II molecules (see section 1.1.3.3). Upon activation, maturing DCs migrate to lymphoid tissues where they can enter T cell-rich areas by virtue of their expression of CD49d β1 integrins and CCR7 (366). Mature DCs upregulate MHC class II expression and co-stimulatory molecules, thereby increasing their T cell stimulation potential (9).

Recognition of pMHCs on mature DCs leads to naïve T cell activation and proliferation as evidenced by a 20-100 fold increase in the number of antigen-specific CD4 effector T cells within secondary lymphoid organs few days following immunization or infection (362;367). As naïve T cells proliferate and differentiate into effectors, their gene expression profile is modulated by changes in chromatin structure and the profile of recruited transcription factors (368-370) CD4 effector T cells can be subdivided into two subsets largely based on the array of cytokines they produce. T helper type 1 (T_H1) cells produce several characteristic cytokines, most notably IL-2 and IFN-γ, promoting the development of a strong cell-mediated immune response particularly effective against intracellular pathogens like *listeria monocytogenes, leishmania major* and most viruses (371). T_H1 cells are characterized by expression of T-bet, a member of the T-box family of transcription factors that acts in part by inducing irreversible chromatin remodelling and expression of the gene encoding IFN-γ (372;373). On the other hand, T_H2 cells predominantly secrete IL-4, IL-5 and IL-13, promoting allergic responses yet effective in eliminating large extracellular parasites such as helminths (374). Factors influencing CD4 T cell differentiation include the nature of the pathogen, the dose of antigen, the route of

entry as well as the genetic background of the host (375;376). These factors will greatly influence the cytokine environment during T cell activation, the most important factor controlling CD4 T cell polarization. For example, the T_H1-inducing cytokine IL-12 is produced mostly by a subset of DCs as well as by phagocytic cells in response to intracellular parasites; IL-12 in turn drives T_H1 polarization which is effective at eliminating intracellular pathogens (377). The generation of T_H2 cells is less clear, but evidence is mounting that their development may be a default pathway in the absence of IL-12 exposure combined with contact with IL-4 (371;378).

Following their priming in the T cell-rich area, a specific subset of Th cells downregulates CCR7, upregulates CXCR5 as well as CCR4 and migrates toward the B cell-rich follicles of secondary lymphoid organs where B cell activation and maturation take place, resulting in the formation of germinal centers (379-382). B cell activation is promoted by a complex array of interactions between B and T cells including T helper cell secretion of IL-2, 4, and 5 and via ligation of CD40 (expressed on B cells) by CD40 ligand (expressed on T cells) (383;384). Activated B cells expanding in the germinal centers of lymph nodes have been shown to undergo genetic changes within their rearranged B cell receptor loci. These are of two types: first, genetic hypermutation of the heavy and light chain hypervariable regions takes place within the Fab portion of the antibody; these regions are directly involved in the recognition of the antigenic epitope (11). Through hypermutation, antibodies with higher affinity for their Ag are generated and preferentially selected, a process called affinity maturation (385;386). Second, antibody isotype switching takes place through somatic recombination of the locus encoding for the constant (Fc) portion of the antibody heavy chain (387). Isotype switching confers the resulting secreted antibodies with different functional properties.

Naive $CD8^+$ precursor CTLs require help from $CD4^+$ T helper cells in order to differentiate into mature CTLs (388). The nature of this help is not completely understood and likely depends, to some extent, on the effect of cytokines such as IL-2 and IL-4 (389;390). Importantly, CD40 ligand can substitute for T helper cells in the generation of CTL (391-393). CD40 ligand is expressed on activated T helper cells, and its blockade

inhibits CTL priming (393). Recent papers highlighted the importance of CD4 T cell help in the generation of high quality, effective memory CD8 T cells (394-397). It appears that CD8 T cell proliferation in response to a second encounter with Ag is severely impaired in memory CD8 T cells formed in the absence of CD4 T cell help. Moreover, cytokine production and survival following restimulation is reduced in "unhelped", as compared to "helped" CD8 memory T cells. These results complement those showing that the loss of CD4 T cell function during acute and chronic infections can have deleterious consequences on the ability of antigen-specific CD8 T cells to maintain their effector responses and to eradicate or control the infectious agent (398) (399-408).

1.1.5.2 CD8 T cell activation during the primary immune response

In the past few years, the major technical breakthrough in generating tetrameric MHC class I-peptide complexes (tetramers) has revolutionized the analysis of CD8 T cell responses (409). These multimeric fluorescent reagents allow the direct labelling of viable antigen-specific effector and memory CD8 T cells; because of their very low frequency, antigen-specific naïve CD8 T cells cannot be detected using tetramers (409;410). Tetramers of mouse, monkey and human MHC class I molecules complexed with immunodominant peptides from a variety of pathogens have been generated, allowing the quantitation as well as the phenotypic and functional characterization of antigen-specific CD8 T cells in several infections.

Similar to CD4 T cells, priming of naïve CD8 T lymphocytes by dendritic cells occurs within T cell-rich areas of secondary lymphoid tissue. Optimal T cell activation results in massive clonal expansion, occasionally resulting in very high frequency (up to 70% of total CD8) of CD8 T cells specific for a single Ag (411;412). At least in LCMV infection, CD8 T cell clonal expansion appears to be faster and more prominent than for CD4 T cells (413). The initiation of proliferation is coupled to changes in function, cell surface phenotype and migration properties. A program of gene expression that arms the

CTLs with an arsenal of cytolytic and noncytolytic effector mechanisms to fight the infection is initiated. These include:

- **Direct cytolysis of infected cells.** Activated CTLs can destroy abnormal cells using two distinct molecular pathways: the granule exocytosis pathway (major) and the Fas/FasL pathway (minor) (414). First, activated CTLs contain in their cytoplasm exocytic granules loaded with cytotoxic molecules, including the membrane-disrupting proteins perforin and granulysin as well as several members of the serine proteases Granzyme (Grm) family (415;416). Upon TCR triggering by antigen-bearing target cells, specific signals are generated in the effector lymphocyte that cause the granules to migrate toward the site of contact, a movement orchestrated by the microtubule-organizing center (MTOC) (417). At the cell surface, the granules fuse with the plasma cell membrane and its contents are secreted into the intercellular junction (synapse) formed between the CTL and its target cell (353;354). There, in the presence of calcium, perforin polymerizes and forms pores into the target cell membrane. The exact function of these pores during CTL-mediate killing is still unclear but it is hypothesized that they induce membrane damages and facilitate granzyme entry into the target cell (415). Perforin-deficient mice are more susceptible to a variety of viral infections and show delayed tumor clearance in whole animal systems (418;419). Surprisingly, experiments using these mice revealed a possible role for perforin in CD8 T cell homeostasis; these mice have three to four-fold increased antigen-specific CD8 T cell expansions following infection, as compared to normal mice (420;421).

  Following their entry into the target cell, GrmA and B trigger apoptotic pathways by cleaving a variety of proteins directly or indirectly involved in cell death. For example, GrmB has been shown to cleave several procaspases (2, 3, 7, 8, 9 and 10), bcl-2 family member Bid and DNA fragmentation factor 45 (DFF45) (415;422;423). Cleavage of these substrates induces DNA fragmentation and ultimately apoptotic death. Indeed, Granzyme B–deficient CTLs are profoundly impaired in their ability to induce DNA fragmentation and apoptosis in target cells (424).

The Fas/FasL pathway is initiated by the upregulation of FasL on activated T cells, which triggers programmed cell death by the aggregation of Fas on target cells. Fas aggregation results in a death inducing signalling complex (DISC) formed by aggregation of the receptors and followed by recruitment of an adaptor molecule, Fas associated death domain (FADD) and procaspase-8 (FLICE) (425). The cleavage/activation of procaspase-8 leads to activation of effector caspase-3 and possibly pro-apoptotic Bid, which liberates several proteins including cytochrome c from the mitochondrial intermembrane space. In the cytoplasm, cytochrome c triggers oligomerization of apoptotic protease activation factor-1 (apaf-1) and the recruitment of procaspase-9; caspase-9 then becomes activated and in turn activates downstream caspases such as caspase-3, 6 and 7, leading to downstream effector molecule activation, DNA damage and ultimately apoptosis (426;427).

- **Secretion of cytokines**. After stimulation through their antigen receptors, CD8 T cells produce cytokines such as IFN-γ and TNF-α. In the case of viral infection, these cytokines are believed to inhibit viral replication by altering gene expression in host cells, thereby influencing expression of host factors absolutely required for viral gene expression (419;428-430).
- **Regulated expression of chemokines and chemokine receptors.** The ability of CTLs to specifically home to sites of infection is critical in order to eliminate invading pathogens. T cell trafficking largely depends on the chemokine/chemokine receptor system. Therefore, depending on the site and/or the type of infection, CD8 T cells will modulate the expression of specific chemokines and chemokine receptors, allowing their selective homing (as well as the homing of other leukocyte subsets) at the site of inflammation (431;432).

1.1.5.3 Termination of the T cell-mediated immune response

The massive proliferation of antigen-specific effector T lymphocytes, combined with the action of other cell types from the innate and adaptive immune system, normally leads to

the complete elimination of the infectious agent. In order to preserve homeostasis of the immune system following an immune response, the majority of clonally-expanded T cells must be eliminated by a process known as activation-induced cell death (AICD) (433). Activated T cells die by two different independent mechanisms. First, the antigen-induced expression of the death-inducing molecules FasL and TNF triggers apoptosis pathways by interacting with cognate cell surface receptors (Fas, TNF receptors 1 and 2) whose expression is also induced by TCR signals (434;435). As mentioned earlier, engagement of these receptors activates the caspases cascade and eventually leads to cell death. It has been shown that proliferating T cells exposed to IL-2 have an increased susceptibility to Fas and TNF killing, probably as a result of a more efficient recruitment of the DISC (433;436). Furthermore, IL-2 can sensitize activated T cells to apoptosis by decreasing the expression of FLICE inhibitory protein (c-FLIP), an anti-apoptotic molecule that inhibits caspase-8 activation by precluding its recruitment to the DISC (437;438).

The second mechanism of AICD is caused by the withdrawal of a "survival" factor when the level of antigen decreases and immune activation declines. Lymphokine withdrawal appears to directly induce cytoplasmic caspase activation and necessitate the release of cytochrome c from the mitochondria (433). Overall, IL-2 is believed to be the key regulator of T lymphocyte AICD, as demonstrated by the abnormal accumulation of activated T cells in mice deficient for IL-2 or IL-2 receptor (439-441). Recent experimental data strongly suggest that IFN-γ also plays a major role in the deletion of CD4 and CD8 T cells following the primary immune response, as evidenced by the fact that activated T cells elimination following bacterial or viral infection is severely impaired in IFN-γ-deficient mice (420;442;443). IFN-γ may mediate its effect on AICD by stimulating the expression of caspases downstream of the Fas death receptor through activation of the transcription factor STAT-1 (443).

1.1.6 T cell memory

1.1.6.1 General considerations

A key characteristic of the acquired response is the establishment of immunological memory, which is defined as a more rapid and more effective immune response directed against a previously encountered pathogen (Figure 4). While they are first called into action during the primary imune response, T cells are selected and undergo maturation processes endowing them with the enhanced functional properties underlying memory responses (444). Despite the massive death of antigen-specific T cells at the end of the primary response, the precursor frequency of memory T cells is much higher than for naïve cells (411;445-450). Memory T cells show a shorter lag time for cytokine secretion, proliferation, acquisition of cytolytic function and migration to infected tissues following Ag exposure; they are also less dependent on co-stimulation and respond to lower doses of Ag (451-457).

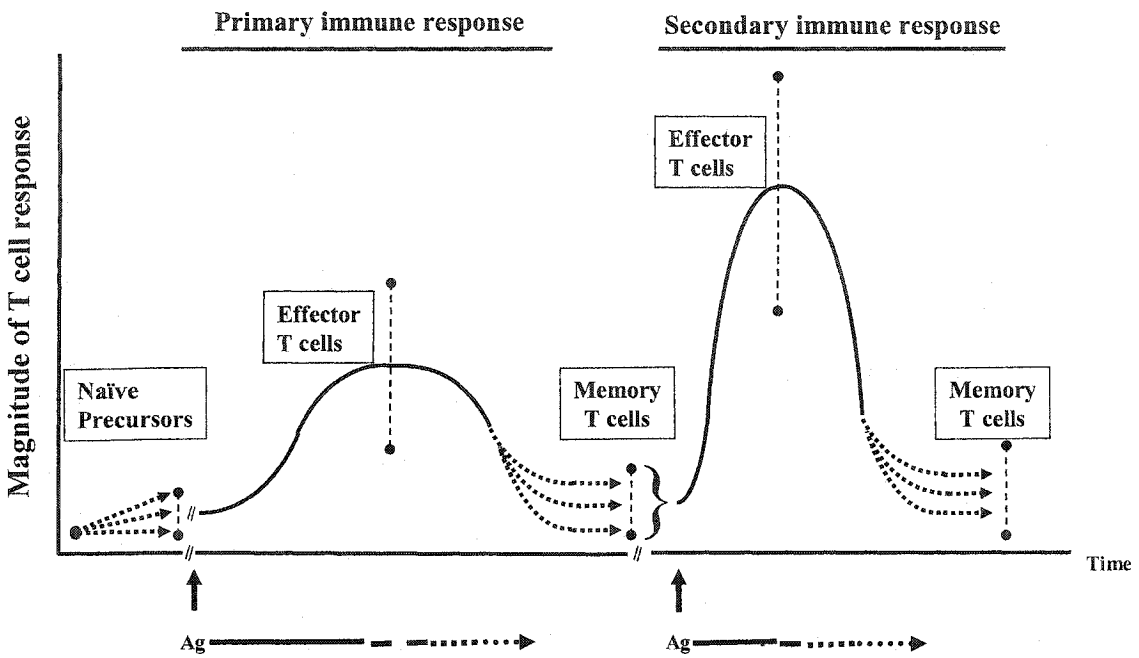

Figure 4. Antigen-specific T cell immune responses. *Naive T cells found in the periphery are derived from single T cell clone precursors (recent thymic emigrants) having undergone low-level homeostatic expansion. Prior to any antigen-driven expansion, specific peptide-MHC combinations are recognized by variable numbers of T cell clones. Upon encounter with their specific antigen, T lymphocytes undergo clonal expansion and constitute the primary immune response. Following the massive T cell expansion, the death phase of the primary response occurs during which effector responses become undetectable. Memory is seeded during the primary immune response and persists in absence of detectable antigen. Re-encounter with antigen results in the secondary response characterized by a rapid expansion of memory T cells. Altogether, memory responses are recruited more rapidly, display stonger effector activity, and resolve antigenemia more rapidly than primary responses.*

1.1.6.2 T cell differentiation subsets

T cells are classified into subsets on the basis of their history and functional properties, and not because they express a specific set of phenotypic markers. As T cells are constantly re-modelled by the various stimuli they meet, the resulting dynamic nature of their differentiation precludes their categorization into statically defined subsets. In fact, at any given time and anatomical site, combinations of commonly used markers serve at best to restrict focus to pools of T cells within which subset-specific properties are

enriched (458). Table II provides an overview of some of the molecules commonly used to dissect T cell subsets.

	Marker	Expression enriched in subsets of	Protein superfamily
Cluster of differentiation molecules	**CD11b***	E	Integrin
	CD25 (IL-2Rα)	E	Cytokine receptor
	CD27	N, M	TNF-Receptor
	CD28	N, M	Immunoglobulin-like
	CD44*	E, M	Cartilage link protein
	CD45RA[T]	N, E	PTP Receptor
	CD45RO[T]	E, M	PTP Receptor
	CD49d*	E, M	Integrin
	CD56	E, M	Immunoglobulin-like
	CD57	E, M	N/A
	CD62L	N, M	C-type lectin
	CD69	E	C-type lectin
	CD95 (Fas)	E, M	TNF-Receptor
	CD95L (FasL)	E, M	TNF
	CD137 (4-1BB)	E	TNF-Receptor
	CD154 (CD40L)	E	TNF
	CD134 (OX-40)	E	TNF-Receptor
	CD152 (CTLA-4)	E	Immunoglobulin-like
Chemokine receptors	**CCR5**	E, M	G-protein-coupled receptor
	CCR6	E, M	G-protein-coupled receptor
	CCR7	N, M	G-protein-coupled receptor
	CXCR3	E, M	G-protein-coupled receptor
	CXCR4	N	G-protein-coupled receptor
Others	**ICOS**	E	Immunoglobulin-like
	Ly6c	M	Ly6
	PNA ligand*	E, M	O-glycan
	1B11 ligand*	E	O-glycan

References: (254;447;459-468)

N: naïve, E: effector, M: memory
PTP: Protein Tyrosine Phosphatase
TNF: Tumour Necrosis Factor
* Denotes markers established for murine T cells only
[T] CD45RA and RO are isoforms of the CD45 molecule

Table II: Enrichment markers for T cell subsets.

Mature peripheral T cells may nevertheless be subdivided into two categories: naïve cells and antigen-experienced cells. Naïve cells are those that have not been stimulated by their specific ligand. They are functionally relatively quiescent, although they undergo low-level homeostatic proliferation (469;470). As discussed earlier, activation of naïve T cells will only occur if they encounter their specific peptide/MHC complexes at the surface of professional APCs able to provide them with adequate co-stimulatory signals (471-473).

Antigen-experienced cells may be subdivided into several functional subsets. Effector T lymphocytes are specific to an antigen which is present and induces them to proliferate (i.e. clonal expansion) as well as to exert effector function through the secretion of cytokines and/or the polarized release of cytotoxic granules (419). The second subset of antigen-experienced T cells consists of memory T lymphocytes that are the key protractors of immunological memory. Memory T lymphocytes differ from activated effectors in that they are characterized by the lack of some major ready-effector functions (474). These include the polarized secretion of cytotoxic granules and the production of certain cytokines (IFN-γ): the induction and maintenance of these functions require sustained TCR engagement by cognate peptide/MHC (466;475). Furthermore, both types of antigen-experienced T cells differ from naive T cells in their enhanced sensitivity for antigen (451;452;454;472;476). This is in part due to the increased expression of signalling components such as $p56^{Lck}$, to a developmentally regulated rearrangement of the topology of their signalling machinery as well as to an increase in lipid rafts (477-481).

Phenotypic differences between T cell subsets not only result from differential protein expression, but also from differential post-translation modification of some of these proteins. In mice, activated and memory cells have been shown to bind peanut agglutinin (PNA) to a greater extent than naïve cells, suggesting a reduced number of sialic acid residues on the cell surface O-glycans of antigen-experienced cells (467). Furthermore,

differential O-glycan modification of mucin-type glycoproteins such as CD43 has been shown to discriminate between effector and memory cells (468).

1.1.6.3 Heterogeneity of the memory T cell population

Recently, two subsets of memory T lymphocytes possessing differential effector function and tissue homing characteristics have been identified both in CD4 and CD8; they have been termed "central memory T cells" (T_{CM}) and "effector memory T cells" (T_{EM}) (482). While T_{CM} cells, through their expression of CCR7 and CD62L, home preferentially to lymphoid organs and display little immediate effector function, T_{EM} cells express tissue homing receptors associated with inflammation and display more ready-effector function. Similar subsets have also been identified in the mouse using CD62L expression (483). Recent work by different groups have provided direct evidence of the different homing characteristics of memory cell subsets, with a preferential homing of effector memory to the periphery and the seeding of a functionally distinct memory T cell subset in the lymphoid compartment (484-486).

A pathway of memory T cell differentiation has been established in humans whereby, following antigen-encounter, T cells proliferate and gain effector properties while undergoing phenotypic changes that modify their tissue homing properties (Figure 5A) (482;487). Antigen-specific cells are recruited into a pre-memory subset possessing a naïve-like phenotype (CD45RA+ CCR7+) before reaching the T_{CM} and T_{EM} maturation stages (CD45RA-CCR7+ and CD45RA-CCR7-, respectively). Ultimately, they may reach a "terminally differentiated" effector stage (T_{EMRA}, CD45RA+CCR7-). However, recent experimental data provided evidence that the T_{EMRA} subset derive from T_{CM} that have undergone cytokine-driven proliferation, in the absence of specific TCR stimulation (488). Progression through these subsets is associated with a loss in proliferative potential in combination with a gain in the potency of effector functions. The prevalence of antigen-specific T cells from each memory subset in an individual may therefore be hypothesized to evolve throughout the progression of immune responses (Figure 5B).

In humans, phenotypic dissection of the virus-specific T cell responses directed against HIV, Cytomegalovirus (CMV), EBV and influenza, as well as tumour-specific CD8+ T cells, suggest that immune responses may yield very different distributions of memory cells (487;489-491;491-493). Multiple parameters including the antigen load, the nature of the antigen, as well as the site of antigen production and immune response may influence the resulting distribution of cells between different memory subtypes, thus highlighting a role for the cell's environment in guiding its differentiation (494-496).

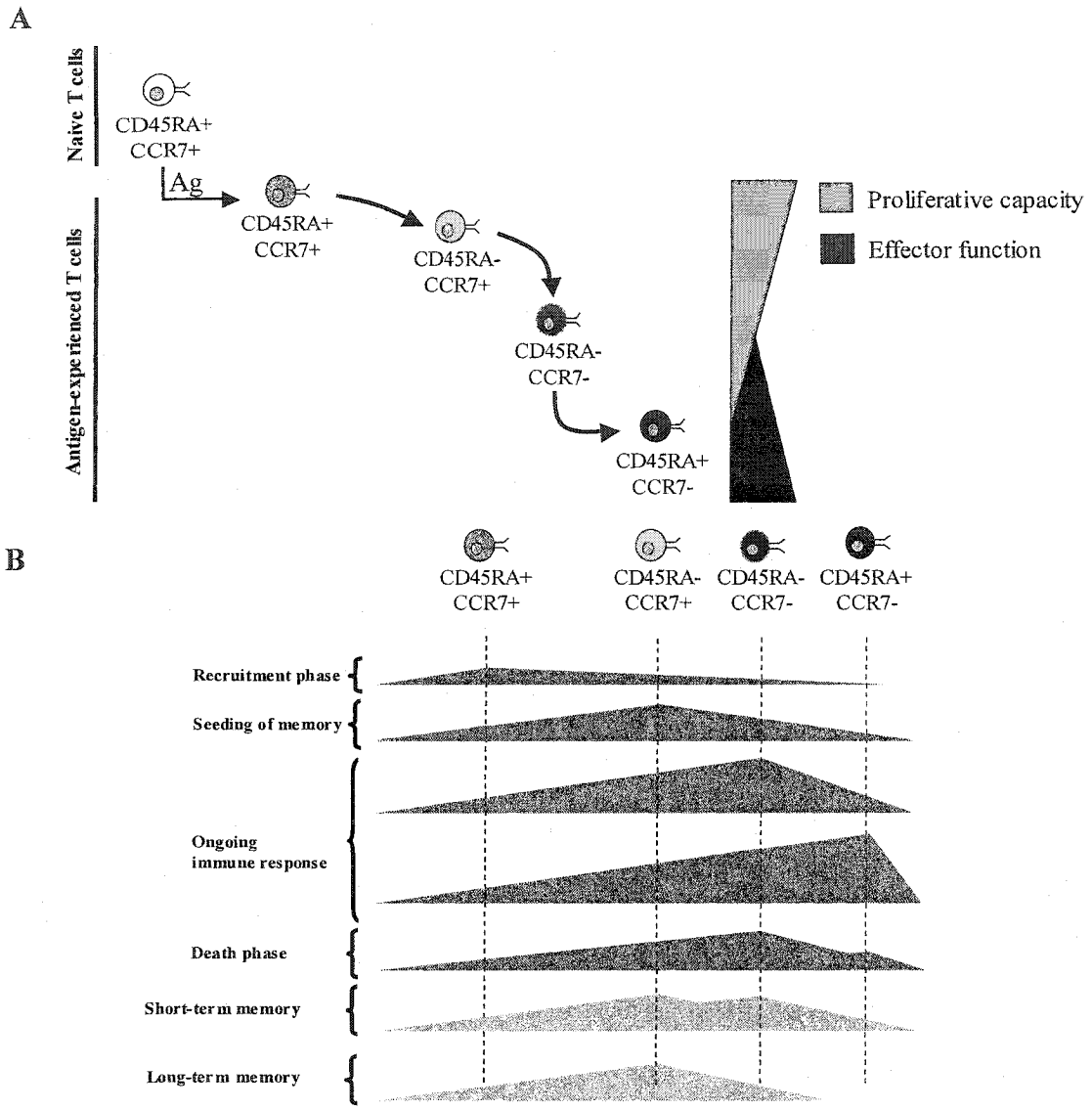

Figure 5. Heterogeneity of the T cell pool. A. *The differentiation of antigen specific T cells results in a heterogenous population of T cells characterized by different proliferative capacity and effector function. Antigen-specific T lymphocytes that are endowed with high proliferative capacity and share phenotypic markers ($CD45RA^{+}CCR7^{+}$) with naïve cells are first recruited into the response. Central memory (T_{CM}) and effector memory (T_{EM}) T cell pools displaying differential phenotypic ($CD45RA^{-}CCR7^{+}$ and $CD45RA^{-}CCR7^{-}$, respectively), functional and homing properties are formed. Differentiated functional effector T lymphocytes are also generated ($CD45RA^{+}CCR7^{-}$).* **B.** *The prevalence of antigen-specific T cells from distinct antigen-experienced subset is hypothesized to evolve throughout the progression of immune responses and may vary due to the nature of the pathogen, the level of antigenemia and the immune (co-stimulation, cytokines) environment. Memory compartments are seeded during the immune response and persist following resolution of detectable antigenemia. Central memory T cells possess stem cell-like properties and are believed to mediate the long-term protection against reinfection.*

1.2 Superantigens

1.2.1 Discovery of superantigens

Toxic shock syndrome (TSS) was first described in 1978 as a major systemic illness associated with non-invasive *Staphylococcus aureus* (*S. aureus*) infection in children (497). TSS is characterized by fever, rash formation, hypotension, vasodilatation, hypoalbuminaemia, acute respiratory distress syndrome and multiple organ failure (498;499). Major interest in the disease was sparked during the early 1980s when a significant number of staphylococcal TSS cases occurred in healthy young women using high-absorbency tampons (500). In 1981, two groups independently characterized a secreted protein called toxic shock syndrome toxin (TSST)-1 from *S. aureus* that was highly associated with TSS in menstruated women (501;502). The agents responsible for TSS in *S. aureus* and *Streptococcus pyogenes* (*S. pyogenes*) infection are a group of potent immunostimulatory exotoxins belonging to the large family of pyrogenic toxins (503;504). The term "superantigen" (SAg) was introduced in 1989 to describe the massive polyclonal expansion of T lymphocytes displaying specific TCR Vβ when exposed to these bacterial exotoxins (505;506).

It should be noted however that superantigen from viral origin (vSAGs) had previously been discovered in 1973 by Hillyard Festenstein and referred to as "minor lymphocyte-stimulating" (mls) antigens (507;508). Notably, these viral SAgs are 45 kD type II transmembrane proteins with no sequence similarity to the bacterial SAgs (509). Mls antigens are now known to be vSAgs encoded by the endogenous murine retrovirus Mouse Mammary Tumor Viruses (MMTV) proviruses that have randomly integrated into germ cells (509). Experimentally, Mls antigens expressed on B cells induce massive T cell expansion in mixed lymphocyte reaction (MLR) between MHC-identical strains of mice (507;510). As such, it is now understood that the vSAg molecule from MMTV is an essential component of the life cycle of the virus, providing efficient viral replication in newly infected B cells by recruiting Vβ-specific T cell help and promoting B cell proliferation (509).

There is little evidence of possible endogenous human SAgs. A provocative and controversial study published in 1997 suggested the presence of endogenous SAgs in the *env* gene of a human endogenous retrovirus (HERV) initially named IDDMK$_{1,2}$ 22 and later found to be identical to HERV-K18 (511). Conrad and colleagues found major expansions of Vβ7+ T cells in the pancreatic islets of patients with type I insulin dependent diabetes mellitus (IDDM) which was attributed to endogenous SAg. However, several independent groups have been unable to reproduce these findings (512-519). Nevertheless, it was recently shown that the EBV-associated superantigenic activity is due to its ability to transcriptionally activate, most probably by the induction of IFN-α secretion, the *env* gene of the endogenous retrovirus HERV-K18, leading to the specific activation of Vβ7+ and Vβ13+ T cells (510;520;521).

1.2.2 Description and classification of superantigens

The work described in this thesis focuses on superantigens from bacterial origin. SAgs are protease-resistant globular proteins of about 20-30 kD produced by a variety of Gram positive bacterial species including *S. Aureus, S. pyogenes*, *Streptococcus dysgalactiae*, *Clostridium perfringens*, the Gram negative bacterial species *Yersinia pseudotuberculosis* and the wall-less *Mycoplasma arthritidis.* The repertoire of *S. aureus* SAgs includes the staphylococcal enterotoxins (SE) serotypes A, B, C (C1, C2, C3), D, E, G, H, I, J, K, L, M, the toxic shock syndrome toxin-1 (TSST-1) as well as the exfoliative toxins ETA and ETB (503;504;522;523). SAgs from *S. pyogenes* includes streptococcal pyrogenic exotoxins (SPE) serotypes A, C, G, H, I, J, L, M, streptococcal superantigen (SSA), *S. pyogenes* mitogen (SPM) and SPM-2, variants of streptococcal mitogenic exotoxin Z (SMEZ, SMEZ-2, SMEZ-3/SPEX) (503;504;522;524). *Streptococcus dysgalactiae*, *Clostridium perfringens*, *Yersinia pseudotuberculosis* and *Mycoplasma arthritidis* produce *Streptococcus dysgalactiae*-derived mitogen (SDM), *Clostridium perfringens* enterotoxin (CPET), *Yersinia pseudotuberculosis*-derived mitogen (YPM) and *Mycoplasma arthritidis* mitogen (MAM), respectively (524). The best-characterized SAgs from staphylococcal and streptococcal origins (collectively known as the pyrogenic

toxin SAg family) can be classified into five subgroups according to their amino acids sequence (table II).

Group	SAgs	MHC class II binding α/β	Human TCRVβ specificity	References
I (TSST-1)	TSST-1	+/-	2, 5.1, 8.1	(508;525)
II (SEB subfamily)	SEB	+/-	1.1, 3.2, 12, 13.2, 14, 15,	(508;526)
	SEC1	+/-	3.2, 6.4, 6.9, 12, 13.2, 14, 15, 17, 20	(526;527)
	SEC2	+/-	12, 13, 14, 15, 17, 20	(526)
	SEC3	+/-	12, 13, 14, 15, 17, 20	(526)
	SSA	+/-	1, 3, 15	(528;529)
	SPEA	+/-	2.1, 12.2, 14.1, 15.1	(530;531)
	SEG	?/?	3, 12, 13.1 13.2, 14, 15	(532;533)
III (SEA subfamily)	SEA	+/+	1.1, 5.2, 5.3, 6.3, 6.4, 7.3, 7.4, 9.1, 16, 21.3, 22, 23	(508;534)
	SEE	+/+	5.1, 6.3, 6.4, 6.9, 8.1	(508;534)
	SEJ	?/?	?	(535)
	SED	+/+	1, 8.1, 9, 21.3	(525;536)
	SEH	-/+	Vα 10, 14, 17	(537-539)
IV (SPEC subfamily)	SPEC	-/+	2	(508;540)
	SPEJ	-/+	2.1, 8.1	(541)
	SPEG	?/+	2.1, 4.1, 6.9, 12.3	(542)
	SPEL	-/+	1.1	(543)
	SPEM	-/+	1.1	(543)
	SMEZ	?/+	2.1, 4.1, 7.3, 8.1	(542;544)
	SMEZ-2	-/+	4.1, 8.1	(542;545)
	SMEZ-3	?/?	8	(546)
	SDM	?/?	1, 23	(547)
V (SEI subfamily)	SEI	?/?	1, 5.1, 5.3, 6, 9, 23	(533)
	SEK	?/?	5.1, 5.2, 6.7	(548)
	SEM	?/?	6, 8 ,9, 18, 21	(533)
	SEL	?/?	5.1, 5.2, 6.7, 7, 9, 16, 22	(533;549)
	SPEH	-/+	2.1, 7.3, 9.1	(542;545)
	SPEI	-/+	5.3, 6.9, 9.1, 18.1, 22	(541)

Table III. Classification of the superantigens. *Because TSST-1 causes most of the cases of staphylococcal TSS and is more distantly related to the other toxins, TSST-1 and its variants form their own group (group I). Group II consists of the SAgs related to SEB and contain a cysteine loop structure separated by 10-19 amino acids. Group III consists of the SEA subfamily and these SAgs contain a cysteine loop structure invariably separated by 9 residues as well as a zinc-binding domain. SAgs from Group IV (SPEC subfamily) contain a zinc-binding domain but not a cysteine loop structure. Group V consists of the SEI subfamily and these SAgs also contain a zinc-binding domain as well as a unique 12-amino acid extension (but no cysteine loop). SAgs primarily involved in clinical TSS (TSST-1, SEB, SEC, and SPEA) are located in group I and II whereas group III contains SAgs implicated in most cases of food poisoning (SEA, SED, SEE). The TCRVβ specificity of each superantigen is also listed.*

SAgs bypass the mechanisms of conventional, MHC-restricted, antigen processing and presentation. They bind directly to the MHC class II molecules as intact proteins and interact with TCRs at sites distinct from conventional peptide-binding regions. By binding to the germ-line encoded Vβ region of the TCR, which show much less structural variation as compared to the randomly-generated hypervariable CDR3 regions of the TCR αβ chains, superantigens can interact with a higher fraction of the T cell repertoire. In a typical antigen-restricted immune response, only a small fraction (less than 0.001%) of the host's T cell repertoire will be activated (450). However, since the number of different TCR Vβ regions in human is restricted to less than 50 and since most of the SAgs can bind more than one Vβ, up to 25% of an individual's T cells can be stimulated by a single SAg (499;550). As a consequence, each superantigen is associated with a characteristic Vβ "signature" (table III); for example, TSST-1 stimulates almost exclusively Vβ2+ (and to a lesser extent Vβ5.1+) T cells (499;525;550). Recent studies provided evidence that some SAgs do not engender a characteristic Vβ "footprint" and a massive T cell expansion. For example, it seems that MAM interacts simultaneously with the Vβ region and with the CDR3 loop of the TCR β chain and consequently does not lead to a TCRVβ-specific T lymphocyte expansion (551-554). Also, it was recently shown that SEH induces a TCR Vα-specific expansion of T cells, without any apparent TCR Vβ bias (539). Skewed TCR Vα repertoires after stimulation with SEA, SEB, SEC and SEE have been previously observed but, as opposed to SEH, these SAgs clearly promote Vβ-specific T cell expansion (539;555-559). Moreover, it appears that some SAgs can also stimulate subsets of γδ T lymphocytes (560-565). Therefore, it implies that massive specific TCRVβ expansion can no longer be regarded as the ultimate criterion defining superantigenicity.

Through their ability to activate T cells specific for self-antigens (auto-reactive T cells), SAGs have been associated with a number of autoimmune disorders including diabetes mellitus, multiple sclerosis and rheumatoid arthritis. SAGs are also believed to play a role in other abnormal immunologic states such as psoriasis, eczema, atopic dermatitis, and

Kawasaki syndrome (499;504;508). It must be noted that no direct clear evidence that SAgs represent the causative agents of these immunopathologies has been provided. Nevertheless, numerous reports strongly suggests that SAgs do play a role in the development and/or the exacerbation of these diseases.

What benefit superantigens serve the bacteria has always puzzled microbiologists and immunologists. The fact that a wide variety of microorganisms express these toxins testifies to the evolutionary advantage associated with superantigen secretion. The main hypothesis is that superantigen secretion provokes the "corruption" of the immune response to infection, thereby promoting carriage and transmission of the invading pathogen. Interestingly, the genes that regulate superantigen expression in *S. pyogenes* also regulate mechanisms of immune evasion such as M protein and capsule expression (499). How might SAgs prevent the normal development of immunity to infection? It is possible that the massive release and comsumption of soluble mediators by superantigen-activated cells hamper the normal activation and trafficking of antigen-specific T cells. Moreover, it is possible that the massive infiltration of superantigen-specific T cells in the T cell-rich areas of lymph nodes limits the access, due to physical space limitations, of antigen-specific T lymphocytes, thereby preventing their proper activation by DCs located in the lymph nodes.

1.2.3 Structure of superantigens

Most of our knowledge about the interaction of SAgs with MHC class II and TCR has come from three-dimensional structure determinations. The crystal structure of several SAgs has been reported during the last ten years: TSST-1 (566;567), SEA (568;569), SEB (570;571), SEC2 (572), SEC3 (573), SEH (538;574), SPEA (575;576), SPEC (577), SPEH (545) and SMEZ-2 (545). Despite marked differences at the amino acid sequence level, structural studies on superantigens have revealed a conserved two-domain architecture (amino- and carboxy-terminal domains) with a long, solvent accessible α-helix spanning the center of the molecule (578). The amino-terminal domain (also called the B domain or small domain) consists of a β-barrel that has an α-helix at one end

(except for TSST-1), resembling the oligosaccharide-/oligonucleotide-binding fold (OB-fold) present in the β-subunit of cholera and pertussis toxin (579;580). The amino-terminal domain of SAgs from group II and III (table III) also contains a highly flexible disulfide loop that appears to be implicated in the emetic properties of these SAgs (578;581). The C-terminal domain comprises a four-stranded β-sheet capped by the central long α-helix and has some structural features of the "β-grasp motif" present in other proteins such as ubiquitin, 2Fe–2S ferredoxin, immunoglobulins and the Ras-binding domain of the serine/threonine-specific protein kinase Raf-1 (580). The conserved structural architecture indicates strong evolutionary pressure to maintain structural integrity and ability to bind MHC class II and TCR but it also appears that antigenic variation between SAgs has been maximized (504;582;583). Most superantigens also possess either one or two zinc-binding site(s) which are responsible for interaction with MHC class II molecules and/or for homodimerization (578).

1.2.4 Interaction of superantigens with MHC class II molecules

The three-dimensional structure of five different SAgs-MHC class II complexes has been determined so far: SEB and HLA-DR1 (584), SEB and HLA-DR4 (585), TSST-1 and HLA-DR1 (586), SPEC with HLA-DR2a (587) and SEH with HLA-DR1 (574). The binding of SEB to HLA-DR1 and HLA-DR4 revealed that SEB binds to the α1 domain of both proteins, away from the peptide-binding groove (584;585). Because the DRα chain is common to all HLA-DR molecules, it explains why SEB can bind to many different DR alleles (588). TSST-1 extends over the peptide-binding pocket of HLA-DR1 and contacts the α-helix of the β1 domain, explaining the influence of the MHC-bound peptide on the binding of TSST-1 (586;589-592). Although the binding sites for SEB and TSST-1 are overlapping, these SAgs do not compete with each other because they bind different subsets of HLA-DR1 molecules (593).

The SPEC/HLA-DR2a crystal was the first structure of a superantigen binding to the high-affinity, zinc-dependent site on MHC class II (587). The bridging zinc ion is

tetrahedrally coordinated by three residues from the C-terminal region of SPEC (His167, His201 and Asp203) and one histidine residue from the α helix of the β1 domain of HLA-DR1 (His81); this residue is highly conserved among HLA-DR, DP and DQ alleles (594;595). The three SAgs residues involved in the zinc binding site align with similar residues in SEA, SED, SEE, SEH, SEI, SEJ, SEK, SPEG, SPEJ and variants of SMEZ-2 (508). Mutation of these residues in SEA, SED and SEH decrease the affinity for MHC class II and inhibit mitogenic activity (537;596;597). The binding of SEH to MHC class II molecules is very similar to that of SPEC, suggesting that all superantigens interacting with MHC class II in a zinc-dependent manner present the superantigen in a common way, which is completely distinct from those employed by SEB and TSST-1 (see figure 6) (574). SPEC and SEH extensively interact with the amino-terminal portion of the peptide bound in the antigen binding pocket of HLA-DR, implying that the nature of the peptide influences the binding of these SAgs (574;587;598). It should be noted, however, that the amino-terminal portion of antigenic peptides display less structural variability than the carboxy-terminal segment, suggesting that SAgs bind a relatively conserved region (in terms of structure) of the antigen recognition surface (598-600). It appears also that SAgs interact mostly with the backbone of the peptide, possibly limiting the effect of peptide side-chains on the interaction and allowing the binding of SAgs to a large proportion of MHC class II molecules.

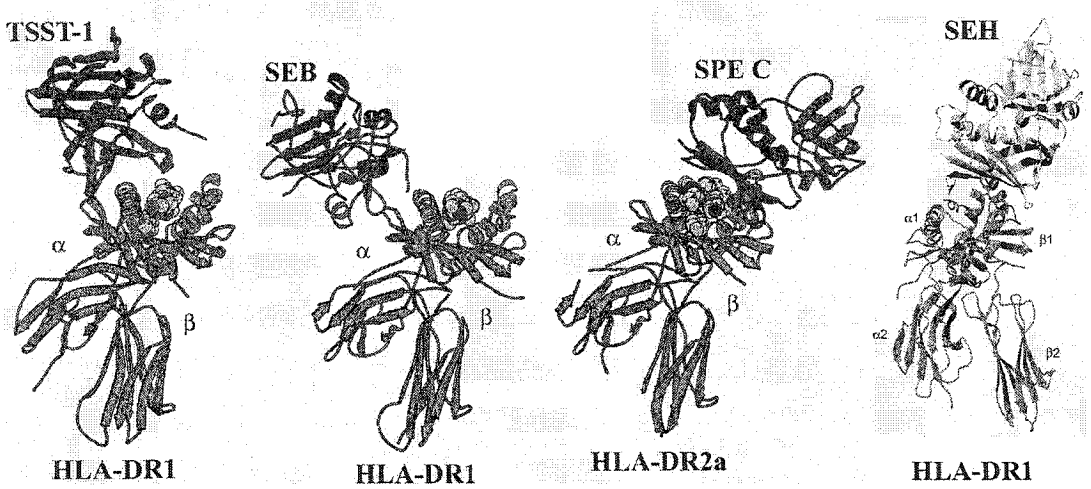

Figure 6. Comparison of the structure of four different SAgs-MHC class II complexes. *MHC class II molecules are represented in green, antigenic peptides are in yellow or in red (in the SEH/HLA-DR1 complex). The superantigens are in blue+red or yellow (in the SEH/HLA-DR1 complex). Adapted from Li et al. Immunity 14: 93-104, Copyright (2001), with permission from Elsevier and from Petersson et al. Embo J. 20: 3306-12, Copyright (2001) with permission from Oxford University Press.*

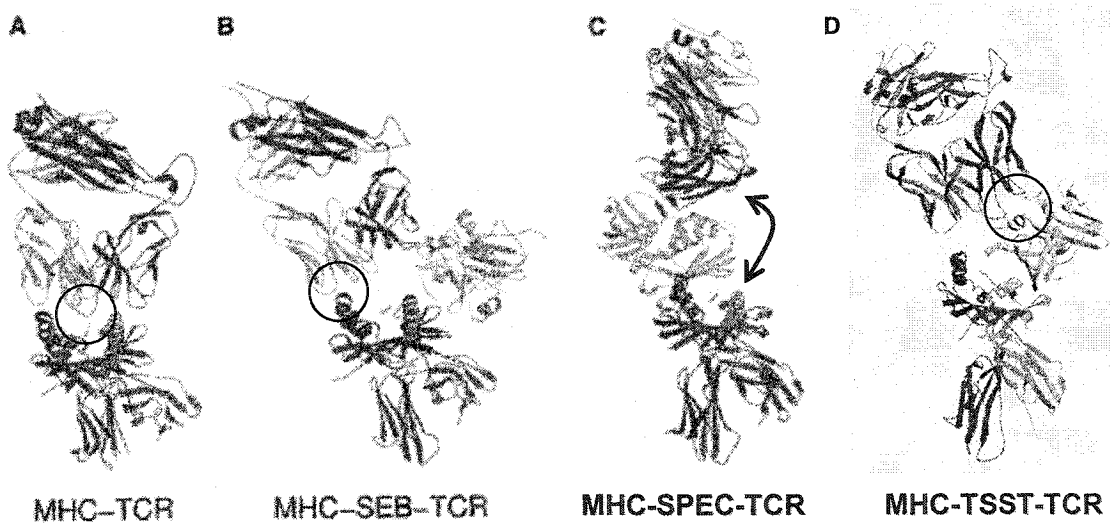

Figure 7. Comparison of the structure of three different ternary TCR-SAgs-MHC class II complexes. **A.** *Structure of a TCR-peptide/MHC class II complex (190). The contact between the TCR CDR3 loops and the antigenic peptide is highlighted (circle).* **B.** *Model of the TCR-SEB/MHC class II complex. The interaction between the TCR α chain CDR2 loop and the MHC class II β chain is highlighted (circle).* **C.** *Model of the TCR-SPEC-MHC class II complex. The TCR is displaced away from its position in the TCR-pMHC complex, which prevents any possible contact between the TCR and the MHC molecule (arrow).* **D.** *Model of the TCR-TSST-1/MHC class II ternary complex. The circle represents the putative interaction between the TCR CDR3 loops and portions of the MHC β chain. The colors are as follow: TCR α chain (orange), TCR β chain (red), MHC α chain (green), MHC β chain (blue), superantigens (yellow) and the antigenic peptide (gray). Adapted from Sundberg et al. Structure 10: 687-699 Copyright (2002) with permission from Elsevier and McCormick et al. J. Immunol. 171: 1385-1392 Copyright 2003 with permission from The American Association of Immunologists, Inc.*

Although the crystal structure of SEA complexed with MHC class II molecules has not been resolved, mutagenesis and binding studies have revealed that SEA possesses two distinct binding sites on MHC class II. A low-affinity (K_D = 1 X 10^{-5} M), SEB-like site on the DRα chain and a high affinity (K_D = 1 X 10^{-7}M), zinc-dependent site on the DR1 β chain (596;601-605). Consequently, SEA can activate the APCs by cross-linking cell-surface MHC class II molecules (606-609). It is known that antibody-mediated MHC class II aggregation leads to activation of several PTKs, translocation of PKC, increased intracellular Ca^{2+} and cAMP as well as translocation of MHC molecules into lipid rafts (610-612); such effects can probably be extrapolated to SEA-mediated MHC class II cross-linking (613-615). Disruption of either MHC binding sites of SEA abolishes induction of cytokine mRNA expression in monocytes and greatly impairs T cell stimulation (589;616). Such MHC cross-linking is believed to augment the potency of SEA by increasing the concentration of MHCs, SEA, as well as ICAM-1 and CD80/CD86 in defined cell-surface area (617-619); these "activation clusters" containing increased concentrations of stimulatory molecules would be very efficient at triggering T cell activation (508).

1.2.5 Interaction of superantigens with the TCR

Much of our current knowledge about the SAgs-TCR interaction comes from the work of Roy Mariuzza and colleagues. The crystal structure of the β chain of the mouse 14.3.d TCR (mVβ8.2, Vβ8.2Jβ2.1Cβ2) complexed with SEC2 (620), SEC3 (620), SEB (wild-type and mutant) (621) and SPEA (622) as well as the human TCR Vβ2.1 (hVβ2.1, Vβ2.1Dβ2.1Jβ2.3Cβ2) complexed with SPEC (622) have been determined so far. SEB and SEC3 interact extensively with residues from the CDR2 loop, the framework region (FR) 3 and, to a lesser extent, hypervariable region 4 (HV4) and FR2 of the TCR; these results are in agreement with previously published mutational evidence for a role of TCR Vβ CDR2 and HV4 regions in SAgs recognition (623-625). Consistent with the finding that most bacterial SAgs stimulate T cells expressing particular Vβ elements without obvious selection for specific CDR3 length or sequence (626), there are no direct contacts

between SEB (and SEC) and the TCR CDR3β region. SEB and SEC residues involved in TCR Vβ recognition are located in both the small and the large domains. These include: Thr18, Gly19, Leu20 (Thr20 in SEC3), Glu22 (SEB only), Asn23, Tyr26, Asn60, Tyr90, Tyr91 (Val91 in SEC3), Phe177 (Phe176 in SEB) and Glu210 (588). Interestingly, residues Asn23, Asn60 and Tyr90 are conserved among mVβ8.2-reactive SAgs (SEB, SEC1-3 and SPEA) and they have been shown to represent "hot spots" for interaction with the mouse 14.3.d TCR β chain (588;627). Most of the hydrogen bonds between SEB and the TCR β chain are formed between SEB side-chain atoms and Vβ main-chain atoms, explaining the ability of these SAgs to interact with a number of different Vβ families (because recognition of main-chain atoms can be highly sequence independent) (588).

As expected from their structural similarities, SPEA and SEB bind the mVβ8.2 in a similar fashion but two structural differences distinguish the SPEA-TCR complex from the SEB-TCR complex. First, an asparagine residue (Asn28) from the CDR1β loop forms a hydrogen bond with a glutamic acid side-chain (Glu94) in the SPEA disulphide loop (622). Second, many hydrogen bonds formed between SPEA and mVβ8.2 directly involve side-chain atoms from both proteins, thereby possibly restricting the Vβ reactivity of SPEA as compared to SEB (598;622).

The crystal structure of SPEC bound to the hVβ2.1 revealed a new mode of interaction, with multiple contacts between the SAg and all three CDR regions of the TCR β chain (622). The hVβ2.1 contains amino acid insertions in the CDR1 and 2 regions as well as a flexible CDR3 loop, allowing the formation of numerous hydrogen bonds and van der Waals interactions with specific residues from SPEC (598;622). Interestingly, SPEC also stimulates Vβ4.1-bearing T cells but with 15-fold less efficiency than Vβ2.1-bearing T cells; this difference is probably explained by the fact that the Vβ4.1 sequence includes the CDR2 insertion but lacks that in the CDR1 loop (540;598).

Although direct binding of soluble TCR β chains to cell-surface, MHC-bound SAgs had been demonstrated more than ten years ago by Gascoigne and colleagues (628), only recently with the development of surface plasmon resonance technology (BIAcore^{TM}) has it been possible to directly measure kinetic constants for TCR binding to SAgs (as well as to pMHC complexes) (272;322;629). The interaction between soluble TCRs (αβTCR or β chain only) and SAgs is characterized by a relatively low affinity (K_D = 10^{-4} - 10^{-6} M) and a fast dissociation rate (k_{off} > 10^{-2} s), similar to the measurements reported for typical TCR-pMHC interactions (275;277;322;556;627;630-632). Such low affinities and fast off-rates were believed to be optimal to serially engage multiple TCRs and for T cell activation (as predicted by the serial triggering model), but mutational studies appear to contradict this assumption. Using mutants of SEB and SEC3 with different affinities for the TCR, a direct relationship was established between the affinity of SAgs for the TCR and their T cell stimulatory potency: the higher the affinity of a mutant of SEB or SEC3 for the TCR β chain, the greater its ability to stimulate T lymphocytes (279;627).

1.2.6 Trimolecular interaction of superantigens with MHC class II and TCR

Although the crystal structure of a MHC-SAg-TCR complex has never been elucidated, models of such complexes have been built based on the available MHC-SAg and SAg-TCR structures (Figure 7) (598). The model for the MHC-SEB (or SEC3)-TCR ternary complex shows that the CDR2 loop of the TCR Vα chain probably makes contact with the α-helix of the MHC β chain; this possibility has been confirmed by biochemical and mutational studies that highlighted the role of the TCR α chain in stabilizing the complex (555;588). In the MHC-TSST-TCR model (633), it appears that the CDR3 loop of the TCR β chain is involved in direct TCR-TSST molecular interactions and could contribute to the TCR Vβ specificity of TSST-1. This model also suggests that the CDR2 loop of the TCR α chain could contact the α-helix of the MHC β chain, similar to the ternary models involving SEB/SEC3.

Distinctly, the ternary complex involving SPEC, a SAgs that binds the β chain of the MHC class II, illustrates a radically different trimolecular topology, whereby the presence of SPEC results in the displacement of the TCR away from its normal position in the pMHC:TCR complex and precludes any direct contact between MHC and TCR.

These models demonstrate that efficient TCR triggering can be achieved by ternary complexes having very different topologies and imply that the geometry of TCR ligation is probably not a critical factor controlling T cell activation (598). The fact that kinetic parameters (K_D, k_{off}) of the MHC-SAg-TCR interaction fall within the range calculated for typical TCR:pMHC complexes strongly suggests that these parameters mainly govern T cell triggering, as predicted by the kinetic discrimination/proofreading model for T cell activation (555;557).

1.3 Project Rationale and Research Objectives

One major obstacle to study the dynamics and properties of antigen-specific human T lymphocytes is related to difficulties in following the most proximal events of the expansion of individual clonotypes due to their low abundance in individuals. Moreover, the specificity of these populations arises from the somatic rearrangement of gene-encoded elements and random addition or deletion of nucleotides, making it difficult to predict the primary structure of individual T cell clonotypes in a way that would allow anticipating and monitoring in real-time their relative distribution in a complex repertoire. To circumvent the limitations of typical antigen-restricted systems, we developed an *in vitro* system making use of bacterial superantigens. Superantigens, by stimulating individual T cell precursors in a specific manner, mimic closely an antigen-mediated T cell response. Moreover, their affinity and kinetics of interaction with the TCR are remarkably similar to antigen/MHC complexes. The work presented in this thesis makes use of this model to study the evolution and the expansion of a CD4 T cell repertoire as well as the functional heterogeneity of naïve and memory CD4 T cell subsets.

The specific research objectives of the work detailed in this thesis were:

I. To identify the TCRVβ families responding to SEA using a flow cytometry-based approach.

II. To study the effect of TCR ligand density on the kinetic of expansion and on the clonotypic diversity of the T cell repertoire.

III. To compare the functional properties of different CD4 T cell clones responding to a specific ligand.

IV. To compare the functional properties of CD4 T lymphocytes with regards to the newly established subsets of central and effector memory T lymphocytes.

V. Using multi-parametric flow cytometry analysis, to identify functionally distinct subpopulations within the previously established memory CD4 T cells subsets.

2 Delayed expansion of a restricted T cell repertoire by low density T cell receptor ligands

A link between the generation of a diverse, high-avidity T cell repertoire and the ability to efficiently eradicate infections or tumours has been previously established (634;635). However, the possible influence of TCR ligand density on the kinetics of activation, on the diversity and on the avidity of the responding TCR repertoire had never been addressed directly. The development of an *in vitro* model making use of a superantigen allowed us to show that presentation of different concentration of TCR ligand directly affects the kinetics of proliferation, the clonotypic diversity and the overall avidity of the responding T cell population.

DELAYED EXPANSION OF A RESTRICTED T CELL REPERTOIRE BY LOW DENSITY T CELL RECEPTOR LIGANDS[1]

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Key words: T cell proliferation, staphylococcal enterotoxin A, antigen presentation, avidity, immune response.

Running title: Diversity of the T cell repertoire selected on low avidity TCR ligands

SUMMARY

The role of TCR ligand density (i.e. the number of antigen/MHC complexes) in modulating the diversity of a T cell response selected from a pool of naïve precursors remains largely undefined. By measuring early activation markers upregulation and proliferation following stimulation with SEA, we demonstrate that decreasing the ligand dose below an optimal concentration leads to the delayed activation of a restricted set of TCRVβ-bearing T cells, with the specific, non-stochastic exclusion of some TCRVβ+ T cells from the activated pool. Our results suggest that the failure of these TCRVβ-bearing T cells to reach the activation threshold at suboptimal ligand concentration is due to the inefficiency of TCR engagement, as measured by TCR internalization, and does not correlate with the relative precursor frequency in the non-immune repertoire. Moreover, even at SEA concentrations that lead to the simultaneous proliferation of all SEA-reactive T cells, we observe marked differences in the ability to secrete cytokines among the different responsive TCRVβ-bearing T cells. Altogether, our results indicate that the development of a T cell response to a scarce display of ligand significantly narrows TCR repertoire diversity by mechanisms that involve focusing of the repertoire on the expansion of those T cells with the highest avidity of TCR engagement.

INTRODUCTION

The recognition of antigens presented by MHC molecules through the TCR is fundamental to the development of an immune response. The diversity of the TCR as generated by somatic molecular rearrangement events is greatest within the distinct CDR3 region which specifically contacts the MHC-bound Ag and, to a much lesser extent, within the gene-encoded variable (V) domain (1,2). Beyond thymic selection, the diversity of the host's T cell repertoire is constantly shaped by antigen-driven expansions and differentiation of antigen-specific T cell precursors, followed by the elimination of a large fraction of the pool of expanded cells by apoptosis (3). These processes contribute to the establishment of an immunological memory that is qualitatively dependent on the diversity of the initial antigen-specific response deployed (4-6). As an example, in disease models such as chronic HIV infection, the clonotypic diversity of the primary CD8+ T cell response against viral antigens constitutes one of the earliest and most significant prognostic factor determining the rate of progression to AIDS (7).

Immune responses can be of quite variable repertoire diversity depending on the particular antigen targeted (8,9). Factors which may affect the diversity of a T cell response include the size of the pool of antigen-responding precursors, as well as the complexity of the antigenic determinant itself (4,10,11). Using tetrameric MHC:Ag reagents, investigators have observed that during an antigen-driven immune response, a strong maturation of the repertoire occurs towards T cell clones with the "best fit" for this epitope (12-14). However, this selective focusing of the T cell repertoire was not observed, for example, in H-2L^d-restricted responses against a lymphocytic choriomeningitis virus (LCMV)-derived epitope (5) and the extent to which a repertoire "focuses" on a narrower diversity of cells might very well depend on antigen-related characteristics that remain to be defined (8,9,15,16). These factors, and the interplay of early cellular events underlying repertoire selection processes need to be better understood.

Given the increasingly recognized importance of the quality of TCR:MHC/Ag contacts in determining the functional outcome of T cell activation, it is possible that the avidity of

this interaction imposes significant restrictions on antigen-driven selection processes despite a remarkable sensitivity of the TCR (12,13,17). A role for TCR ligand density in modulating the potential diversity of a selected T cell repertoire has been previously proposed based on circumstantial evidence obtained with distinct endogenously processed peptides (15,16). However, this has never been directly demonstrated. One major obstacle is related to difficulties in following the most proximal events of the expansion of individual antigen-specific TCR clonotypes due to their extremely low abundance in non-immune individuals (3,18). In addition, the interpretation of these results has been further hampered by the difficulty in controlling the avidity and stability of single epitopes *in vivo* (19).

In order to overcome these limitations, we have developed an approach which employs TCR ligands that are recognized by less variable determinants of the TCR, thereby selecting for a broader frequency of responding precursors. Superantigens (SAgs) interact with the V region of the TCR β chain (20,21), whose primary structure is predictable (e.g. because gene-encoded) and against which a wide panel of specific anti-Vβ antibodies are available (22). Moreover, although the interaction of MHC:SAgs with the TCR largely depends on the structure of the TCRVβ domain, its affinity and kinetics for TCR (23-25), as well as its ability to form an immunological synapse (26), are remarkably similar to MHC:antigen complexes. Also, their binding to MHC class II molecules can be controlled with predictable affinity, avidity and stability (27,28). The division history of individual precursor T cells was tracked using a specific fluorescent dye (29), in order to determine their distribution and kinetics of expansion within the population. Altogether, our results provide a framework to understand the influence of affinity/avidity differences in TCR:ligand interactions on the dynamic of T cell repertoire selection processes and on the generation of highly diverse versus narrow immune responses.

MATERIALS AND METHODS

Reagents and antibodies

The phycoerythrin (PE)-conjugated anti-Vβ1 (IM2355), anti-Vβ2 (IM2213), anti-Vβ5.3 (IM2002), anti-Vβ9 (IM2003), anti-Vβ16 (IM2294), anti-Vβ21.3 (IM2050), anti-Vβ22 (IM2051), anti-Vβ23 as well as the FITC-conjugated anti-Vβ1 (IM2406), anti-Vβ2 (IM2407), anti-Vβ3 (IM2372), anti-Vβ5.2 (IM1482), anti-Vβ13.1 (IM1554), anti-Vβ13.6 (IM1330), anti-Vβ17 (IM1234), anti-Vβ20 (IM1562), anti-Vβ21.3 (IM1483), anti-Vβ22 (IM1484) were purchased from Coulter/Immunotech (Miami, FL). The PE-conjugated anti-CD69 (Leu-23) and anti-CD25, as well as the PerCP-conjugated anti-CD4 (Leu-3a) antibodies were purchased from Becton Dickenson (San Jose, CA). The unconjugated anti-Vβ5 (MH3-2), anti-Vβ5.3 (421C1), anti-Vβ6.7 (OT145), anti-Vβ8 (JR2), anti-Vβ9 (MKB1), anti-Vβ12 (SC511), anti-Vβ23 (HUT78) were obtained from various non-commercial sources. SEA was obtained from Toxin Technology (Sarasota, FL).

Human PBLs purification and [^{3}H]-thymidine incorporation assays Peripheral blood obtained from healthy DR1 blood donors was diluted (1:1) in PBS and underlayered with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) at room temperature. After centrifugation, the interface was collected and washed 3-4 times in PBS/2% FCS and resuspended in RPMI 1640 (GIBCO Laboratories) supplemented with 10% fetal calf serum to yield PBMCs. CD25 and CD69 positive cells were depleted from total PBMC by magnetic cells sorting using the MACS system. (Mitenyi Biotec, Gladbach, Germany). Total PBMC were stained with anti-CD25 (BD347647) and anti-CD69 (BD347823) purchased from Becton Dickenson (San Jose, CA) and washed with degassed PBS/2mM EDTA (loading buffer). Cells (total=10^8 cells/column in 500 μL loading buffer) were incubated at 4°C for 30 minutes with MACS Goat Anti-Mouse IgG MicroBeads (30 ul/10^7 cells), washed again and purified on a MACS RS+ separation column and adapter (cat# 413-01) using a VarioMACs magnetic field. The purified cells were then checked for purity by immunofluorescence and resuspended in RPMI 1640 (GIBCO Laboratories) supplemented with 10% fetal calf serum. For [^{3}H]-thymidine incorporation assays, PBMCs were cultured at 37°C in complete RPMI medium supplemented with 5% FCS in

the presence of SEA for 3 days in round-bottomed 96-well plates. Following incubation, 1 μCi [^{3}H]-thymidine was added for 16 hours at 37°C. Cells were harvested and [^{3}H]-thymidine incorporation was measured using a β-plate counter (Pharmacia, LKB Biotechnology AB).

Intracellular cytokine production

Intracellular IL-2 was measured after 8 hrs of stimulation using procedures described (30). In brief, PBMCs ($2x10^6$/ml) were incubated with either no stimulus, PMA (25 ng/ml) and Ionomycin (1 μg/ml), or SEA for 8 hours. Brefeldin A (10 μg/ml) was added for the final 6 hours of stimulation. After stimulation, cells were lysed, permeabilised and stained with either anti-IL-2 or anti-IFN-γ, anti-CD69 and either anti-CD4 or anti-TCR. Antibodies, isotype controls and lysing and permeabilising solutions were purchased from Beckton Dickenson (San Jose, CA). Control antibody for permeabilisation was purchased from Medicorp (Montreal, PQ).

5,6-carboxylfluorescein diacetate succinimidyl ester (CFSE) labeling and SEA proliferation assays

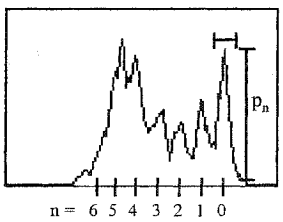
CFSE was obtained from Molecular Probes (Eugene, Oregon, USA). CFSE was dissolved in anhydrous reagent grade DMSO, sealed under nitrogen and stored desiccated at -20 degrees celsius. It was determined that each new preparation of CFSE must be titrated to obtain optimal staining results. Briefly, an equal volume of concentrations between 0.5 - 5 μM CFSE was added to $2x10^7$ PBMCs and incubated with gentle mixing at room temperature for 10 minutes. The reaction was quenched by addition of an equal volume of FCS and cells were washed 3 times with PBS containing 5% FCS. The cells were cultured at 37°C at 5% CO_2 at $1.5x10^6$/ml in RPMI containing 3% FCS overnight and the fluorescence intensity of cells treated with various concentrations determined by FACS analysis. The optimal CFSE concentration was defined to be the one at which all cells were stained with fluorescein at a fluorescence intensity between 10^3 and 10^4 log units in flow cytometry. It was determined that the fluorescence intensity decreases significantly between the time of staining and the next day, so all titration results and experimental

data were obtained only after 24 hours of incubation at the above mentioned conditions. In our experiments, the CFSE concentration used varied between 0.5 to 1.25 μM CFSE.

For cell division assays, CD69-/CD25- PBMCs (1 - 2.5 x 10^5) were cultured with SEA or PHA in 1 ml in 24-well plates. Cells were analyzed every 12 hours by staining with anti-CD4-PerCP and either anti-Vβ-PE or anti-CD25-PE antibodies on ice and compared to non-stimulated cells. All analyses were gated on live CD4+ T cells. CD69 expression was measured after 24 (at 100 pg/ml to 1 ng/ml) or 48 hrs (10 pg/ml and below) and the levels of CD69 expression were compared to non-stimulated cells stained with anti-CD4-PerCP and anti-Vβ-FITC. Flow cytometry analyses were performed on a FACScan using CellQuest software (Beckton Dickenson). For analyses involving anti-Vβ antibodies, at least 150,000 events were acquired for each condition. The average cell division number (ADN) was calculated by taking the peak number of events (p) within each division peak (n) from n = 0 to n = N^{th} division, according to the following equation:

$$ADN = \frac{\sum_{n=0}^{N}(p_n \cdot n)}{\sum_{n=0}^{N}(p_n)}$$

ex:



TCR down-regulation

The human EBV-transformed B cell line LG-2 (DR1/DR1) was provided by Larry Stern (Massachussetts Institute of Technology) and has been described earlier (27). LG-2 cells (2 X 10^5) were pre-incubated with twice the indicated concentration of SEA (in 100 μL) for 3-4 hours in 96-well round-bottom plates to allow the toxin to bind to MHC class II molecules in solution equilibrium. Human PBLs were roughly obtained by incubating PBMCs for 30 min. to 1 hour at 37°C in Falcon culture dishes (to sort out the macrophages by adherence) and collecting the non-adherent cells. Human PBLs (2x10^5) were then added in a final volume of 200 μL and T:APC conjugates were formed by gently centrifuging the plates. The conjugates were incubated at 37°C for 4 hours, after which TCR internalization was stopped by quick incubation on ice. Cells were stained on

ice using a PE-conjugated anti-CD4 antibody (Becton Dickenson, San Jose CA) and the relevant anti-Vβ antibody, and analyzed by flow cytometry on a FACScan using the Cell Quest software (Becton Dickenson, San Jose, CA). The percentage of Vβ-specific internalization was determined by calculating the ratio of the mean fluorescence of SEA-stimulated cultures over the mean fluorescence of unstimulated cultures after 4 hours incubation.

RESULTS

Early activation markers upregulation following stimulation with different concentrations of SEA. In our experiments, we have used the superantigen staphylococcal enterotoxin A (SEA) produced by *Staphylococcus aureus* and which binds the human MHC class II molecule HLA-DR1 with high affinity (31). T cell activation by SEA can be evidenced by measuring the induction of the CD69 activation marker as well as the up-regulation of the IL-2 receptor α chain (CD25). The details of the kinetics have been described elsewhere (28). CD69 and CD25 expression constitute the earliest and least stringent markers for T cell activation and results, including our own, have shown that their expression on T cells represents a reliable marker of previous cognate antigen exposure (own unpublished data and (32,33)). CD69 and CD25 expression was measured 24 and 48 hours following exposure to ligand. These two time points were chosen based on a preliminary determination of the timing of peak marker expression at each of the concentrations tested. Under increasing concentrations of SEA, upregulation of these markers follows a typical progressive dose-response curve similar to the response to any other typical TCR ligand (fig. 1A). At the lowest concentration tested (0.01 pg/ml), we could detect a significant induction of CD25 and CD69 in a small fraction (~ 5%) of CD4 T cells. Of note, our preliminary observations indicate that most likely the same conclusions apply to CD8+ T cells unless otherwise stated (data not shown). As the dose was increased up to 1 ng/ml, additional T cells reached the activation threshold. Importantly, higher doses of SEA (e.g. 100 ng/ml) did not lead to a significant increase in the fraction of CD4 T cells expressing CD25 and CD69 (data not shown).

It is well described that SEA can activate T cells bearing different TCRVβ families. We next wanted to determine if the decrease in the proportion of CD25- and CD69-expressing cells at lower SEA doses is due to the specific exclusion of certain Vβ from the activated pool or to a general failure of T cells bearing different Vβ (i.e. stochastic) to reach the activation threshold. To discriminate between these two non-exclusive possibilities, we examined the TCRVβ-expression of SEA-activated T cells using a wide panel of antibodies available. We measured the peak expression (after 24 hours) of the CD69 activation marker in response to an optimal dose of SEA (1 ng/ml; 10^{-11} mol/l). This analysis allowed us to identify a majority of the SEA-responsive TCRVβ families against which monoclonal antibodies are available (TCRVβ1, 5.2, 5.3, 9, 16, 21.3, 22, 23) (fig.1B). As shown in figure 1C, decreasing the concentration of SEA to 0.5 and 0.01 pg/ml leads to the selective exclusion of some Vβ from the activated (i.e. CD69+) population. For example, whereas Vβ22+ T cells express CD69 at all the concentrations tested, Vβ9+ T cells failed to upregulate the activation marker when the dose is reduced down to 0.01 pg/ml. We obtained similar data by measuring TCRVβ-specific CD25 upregulation (data not shown).

T cell stimulation by a suboptimal ligand avidity leads to a slower onset of activation and to the recruitment of lower numbers of precursor T cells in the proliferative pool. We next wanted to determine if the dose-dependent exclusion of T cells from the activated pool was also occurring when assessing T cell proliferation. Similar to CD25 and CD69 expression, T cell proliferation follows a typical dose-response curve. Indeed, SEA concentrations as low as 0.001 to 0.01 pg/ml (10^{-15}-10^{-14} mol/l) were sufficient for T cell activation whereas maximal stimulation occurred at an optimal dose of 1 ng/ml (10^{-11} mol/l) (fig. 2A). Within this range of concentrations, the number of SEA molecules presented to T cells is linearly related to the concentration of SEA in solution (28).

In the following experiment, human T cells were stimulated, in the presence of autologous antigen presenting cells, by increasing doses of SEA ranging from a minimal (0.01 pg/ml) to an optimal (1 ng/ml) concentration. The kinetic of cell division was measured in activated (CD25+) CD4+ T cells by CFSE dilution. These experiments were

performed under conditions where an excess of IL-2 (500 U/ml) was added to the culture medium, in order to avoid limiting cytokine effects resulting from the insufficient number of IL-2 producing cells at lower SEA concentrations. First, results presented in figure 2B clearly establish that the onset of cell division, for individual T cell precursors, was slower with decreasing doses of SEA ligand. In order to get a more objective indication of the progression of cell division in activated T cells, a population-based Average Division Number (ADN) was calculated from the CFSE intensity of each individual flow cytometry event acquired (see materials and methods for details). Data illustrated in fig. 2C enable us to determine that the average timing of the first division event in cells was significantly delayed (about 36 hours) when stimulating with a minimal SEA concentration of 0.01 pg/ml as compared to stimulation using an optimal dose. The timing of cell cycle entry is thus directly dependent on the amount of ligand encountered by the T cell and correlates with the timing of induction of other T cell activation markers (data not shown). Importantly, figure 2C shows that only the very first division event was delayed at lower doses, as subsequent division events ($n > 1$) occurred at a remarkably similar rate regardless of the initial amount of antigen. This conclusion is based on the comparison of the slopes of the ADN plotted as a function of time for each condition. These results suggest that once cycling, activated T cells divide in an "antigen-independent" manner, an interpretation that is consistent with earlier data demonstrating that the rate of T cell division is limited only by the kinetic of the first division event (34) (35). The average doubling time was 18.5 hours ± 7.8 hours on a Gaussian distribution, corresponding to about 1.3 division/day. As such, it is conceivable that a delay of about 36 hours, which would correspond to about 2 divisions when comparing the lowest concentration to the optimal concentration of SEA (fig. 2C), will have a noticeable effect on the peak amplitude (i.e. at least a 4-fold variation in the total cell number) of the response burst following an exponential division process.

Narrowing of the T cell repertoire diversity selected on a suboptimal ligand concentration. The expansion of different TCRVβ families was next studied under conditions of suboptimal doses of SEA, ranging from 0.01 pg/ml to 1 ng/ml. Given the low, but readily detectable, abundance of some of these Vβ families, we took care to

obtain a homogenous population of resting T cells by depleting pre-activated (CD69+/CD25+) T cells from the population (see materials and methods). Indeed, we have observed that about 1 % of T cells expressed either of these markers in a typical, freshly isolated sample of human peripheral blood lymphocytes (unpublished data from the authors). A typical example is shown in figure 3A and compares the response of TCRVβ1 with that of TCRVβ22, to different concentrations of ligand. Indeed, selection of the repertoire by low doses of TCR ligand, this time led to an asynchronous expansion of the different TCRVβ populations, as opposed to expansions obtained at an optimal dose (see above and fig. 3A at 1 ng/ml). In figure 3A, the time of recruitment of Vβ22+ T cells into cell cycle was already maximal at 1 pg/ml whereas a 1000-fold more SEA was required to trigger a similarly fast response in Vβ1+ T cells. Significant proliferation of TCRVβ1+ cells could not be detected below an SEA concentration of 1 pg/ml when the population was followed for up to 10 days (data not shown). Most likely, SEA concentrations below 1 pg/ml were insufficient to break the activation threshold of Vβ1+ cells. Consequently, stimulation with SEA concentrations as low as 0.05 pg/ml led to a prompt cell cycle entry of TCRVβ22, but no significant division was detected in TCRVβ1 cells.

The behavior of individual TCRVβ families, in fact, mirrors that of the entire population of responding T cells described in the results of figure 2. Indeed, all TCRVβ families proliferated at the same invariable rate once in cell cycle. However, the onset of the first division was delayed to an extent that was directly dependent on the amount of ligand encountered. In the next series of experiments, the results of several reproducible kinetics were analyzed using the same methodology described in fig. 2C and the timing of the first division event (i.e. the time when more than 50% of cells having divided at least once) was determined for each condition within different TCRVβ subsets. As shown in figure 3B, TCRVβ1 cells did not start to divide until a minimal SEA concentration of 1 pg/ml was reached. Remarkably, at increasing ligand concentrations, the first division event within Vβ1 T cells was still significantly delayed relative to other TCRVβs, and this, until the optimal amount of ligand (i.e. 1 ng/ml) was reached. In contrast, TCRVβ22+ and

Vβ23+ cells were recruited in the dividing pool at a concentration as low as 0.01 pg/ml and the onset of division was nearly maximal at concentrations of 0.05 pg/ml. Similar observations were made within other TCRVβ subsets tested, albeit each with a different threshold of activation. These results illustrate two distinct effects of ligand avidity on the composition of the immune repertoire. First, as the amount of ligand is limiting, only cells having a lower activation threshold (high functional avidity) are recruited in the response, resulting in a narrowing of the potential diversity of the selected T cell repertoire. Secondly, as the ligand concentration is increased to break the threshold of activation of individual T cells, non-dividing potential responder cells (i.e. those with highest activation thresholds or low functional avidity) eventually get recruited in the response, but their entry in the dividing pool is delayed. This leads to an "expansion drift" phenomenon by which the relative representation of each responder will change over time until all the potential responders, for that particular concentration have gone through their first division event. These effects are curtailed when an optimal TCR ligand concentration is reached, giving rise to a broader repertoire expanding synchronously and which includes the potentially reactive T cell clonotypes. This is the first direct demonstration of the existence of a hierarchy in the sensitivity of different TCRVβs for a SAg. This hierarchy most likely reflects different functional avidity of each SEA-reactive human TCRVβ family cells and is, in order of decreasing sensitivity: Vβ22 > Vβ23 > Vβ5.3 > Vβ16 ≈ Vβ9 >> Vβ21.3 >> Vβ1.

Synchronous T cell expansion but different cytokine secretion ability of the T cell clonotypes at an optimal ligand dose. Under conditions of optimal amount of SEA ligand (i.e. 1 ng/ml), the onset of the first division occurred at remarkably similar times in each of the different responsive TCRVβ subsets, regardless of possible differences in TCRVβ affinity for the SAg. This rate was also comparable to the levels of stimulation achieved by PHA (see fig. 2B) and likely reflects a constant and invariable average doubling time for activated T cells, consistent with previously published data (29). Under these conditions, the diversity of the repertoire selected remained broad and expansion of individual TCR Vβ families occurred synchronously as determined from comparing the CFSE profiles of each different Vβ family after up to 10 days post-stimulation (fig 4A

and data not shown). In other words, no significant difference in the proliferative rate was observed in expanding SEA-responding TCRVβ family one relative to another, suggesting a remarkable stability of the repertoire selected following stimulation with an optimal concentration of ligand.

It is well established that the activation threshold to elicit diverse effector functions are different. For example, cytotoxic killing, CD25 and CD69 upregulation are triggered at much lower antigen concentrations and by weaker ligands as compared to proliferation and cytokine production (32,33,36). Moreover, it has been shown that the threshold for IL-2 secretion is higher than for T cell proliferation (37-39). To verify if the different responsive TCRVβ reach similar activation thresholds following stimulation with SEA, we compared their capacity to produce IL-2 by intracellular staining. As illustrated in figure 4, a much higher fraction of TCRVβ22+ T cells secrete IL-2 as compared to the other TCRVβ+ T cells. The hierarchy established among the TCRVβ families (see fig. 3) is also maintained when comparing IL-2 production, with Vβ22 > Vβ5.3 > Vβ21.3 > Vβ1. Interestingly, even at the "optimal" (1 ng/ml) SEA concentration (i.e. at which all responsive TCRVβ proliferated synchronously), large differences in the frequency of cytokine-producing cells are observed between the different TCRVβ cells. These results suggest that immediate IL-2 secretion requires a stronger TCR stimulus as compared to proliferation and demonstrate that proliferating T cell clones differ in their ability to mediate effector functions.

The ligand avidity activation threshold for each TCR is primarily determined by the efficiency of TCR engagement. In previous studies, it was established that the degree of TCR internalization is a reliable correlate of the avidity of TCR:ligand interactions (40-43). We compared kinetics of TCR down-regulation in CD4+ T cells within different TCRVβ subsets, in order to verify whether the lack of recruitment of some cells in the proliferative pool correlated with the efficiency of TCR engagement. In the following experiments, TCRVβ1, 21.3 and 22 were tested because they are easily detected, as they are more abundant and thus easier to follow, in a typical human CD4+ repertoire (see fig. 1B). Also, they are representative of the continuum of responses to different SEA

concentrations (see fig. 3B). In all SEA-responsive TCRVβs tested, the internalization of TCRs progressed homogeneously in greater than 85 % of the cells, within the first hour and then remained stable for at least 24 hours (data not shown). Remarkably, a smaller dose of SEA was required to induce a very strong and stable internalization in Vβ22+ T cells (fig. 5). This TCRVβ family indeed has the lowest activation threshold for SEA (see fig. 3). In contrast, little TCR down-regulation was detected in the subsets having the highest activation threshold, namely Vβ21.3 and Vβ1, even at the highest concentration of SEA (about 25% TCR internalization with 100 ng/ml). No significant TCR internalization was detected in the TCRVβ2+ cells that do not proliferate in response to SEA (see fig. 3B). The correlation between the massive TCR down-regulation in Vβ22+ T cells and their ability to secrete IL-2 is in agreement with previously published results demonstrating that cytokine production is only triggered in T cells that show substantial TCR internalization (32,44). Therefore, the efficiency of TCR engagement appears to be a strong correlate of the ability of each TCRVβ subsets to reach activation and of the hierarchy of response of these cells to increasing doses of ligand.

DISCUSSION

In this paper, we dissect the relationships between timing, amplitude and diversity of individual expanding T cell populations evolving within a nascent T cell response triggered by limiting amounts of TCR ligand. The use of SAgs has allowed us to target a higher frequency of expanding precursors since the structure of the TCRVβ region recognized by SAgs is predictable and much less variable, thus avoiding sensitivity problems. But most importantly, we can anticipate T cell populations with predictable specificity, such that they can be probed using available anti-TCRVβ antibodies (22).

Using this system as a model has enabled us to make novel interesting observations. Herein, we directly demonstrate that the density of ligands displayed on the surface of APCs strongly impacts on the size as well as the potential diversity of the response selected from a pre-established set of antigen-specific T cell precursors. Cells having the

highest avidity for that particular ligand dominate the T cell repertoire and the hierarchy of selection is most likely determined by the efficiency of TCR engagement, independently of the distribution of these populations (the percentage of each TCRVβ in healthy donors) in a non-immune repertoire. We did not observe any correlation between the frequency of TCRVβ cells in a non-immune repertoire and their position in the hierarchy of response to increasing doses of ligand. In fact, populations such as TCRVβ1+ cells are abundantly represented in the SEA-responding population, in proportions comparable to TCRVβ22+ cells (see fig. 1B), despite a dramatic difference for their sensitivity of activation. In contrast, TCRVβ23+ is one of the least abundant populations and yet, is one of the most sensitive to small amounts of SEA (fig. 3B). Of note, as is the case for TCRVβ22+ cells, TCR down-regulation in TCRVβ23+ cells was indeed very prominent (data not shown). A competition effect due to the relative abundance of T cell precursors competing for a limited surface of antigen presentation will most likely occur, especially when competing T cells have comparable avidity for the ligand. However, the hierarchy of recruitment of each T cell population in the response pool will mostly depend on the relative efficiency of TCR engagement leading to full T cell activation (42,45,46).

Our interpretation is based on the differential kinetics and extent of TCRVβ down-regulation which have been shown to directly correlate with the TCR:ligand affinity and with the biological outcome, both in antigen and superantigen models (40-43,47,48). Using different murine T cells models, it has been demonstrated that T cells compete for access to MHC/peptide complexes at the surface of APCs (49-52). This competition favors the activation and expansion of high affinity T cells, especially when the number of complexes is low (17,49,53,54) and may explain the lack of direct correlation between naïve T cell precursor frequency and the distribution in the activated pool following T cell proliferation (50,55). Moreover, the T cell-driven down-modulation of MHC/ligand complexes at the surface of APCs has been shown to contribute to inhibiting the response of lower affinity T cells (45,56,57). The massive TCR internalization observed in the Vβ22+ T cell population (see fig. 5) will most likely reduce the number of MHC:SEA complexes at the surface of APCs, thereby limiting the availability of ligands for the

lower affinity T cells (such as Vβ1+ T cells). This phenomenon of ligand depletion could delay or even inhibit the response of low affinity T cells, especially when the number of MHC:SEA complexes at the surface of the APCs is low. According to our observations, the extent to which a response focuses on high avidity T cells will directly be dependent on the availability of the MHC/ligand; this is in agreement with experiments showing an inverse relationship between the antigen dose and the affinity of the T cell population following a primary response (17).

We establish a set of principles that defines the execution of cellular events leading to the selection of an immune repertoire. First, under conditions of optimal ligand stimulation, each of the different T cell populations is recruited in the selected repertoire with comparable kinetics despite differences in their avidity for the ligand. Secondly, all activated T cells divide at a constant and invariable rate that was determined to be approximately 1.3 division/day, regardless of stimulating ligand concentration or TCR affinity. According to our results, the dominance of a T cell precursor was accounted only by the timing of recruitment in the dividing pool and not by differences in their rate of expansion.

Lastly, we observed that both the timing and frequency of recruitment of cells in the dividing pool are directly dependent on the amount of ligand exposure, until optimal concentrations are reached. Clearly, the frequency of a T cell clonotype at the peak of the response will depend on the time at which a naive precursor first encountered its cognate antigen. This was proposed in a number of studies, including one which addressed the timing of a response to temporally distinct exposures to a single antigen (55,58). How can a simultaneous exposure to the same antigen distinctively affect the expansion of two structurally different T cell precursors? We have defined two processes and distinguished them by using the terms "expansion drift" and "activation bias", because they clearly have a different impact on either the distribution or diversity of an immune response. These two processes, which are readily understandable in the context of currently proposed models of T cell activation, are of course clearly functionally related. Indeed, the avidity-related differences in the kinetics at which cells enter the proliferative pool (herein

referred to as expansion drift) likely reflect the longer time interval required to sequentially engage a particular number of individual TCR molecules necessary to overcome a given activation threshold or to stabilize the immunological synapse (59,60). Essentially, the contribution of this first mechanism alone will influence the temporal distribution of the repertoire but will not have a significant impact on the final diversity of the response as all the potentially responsive cells will eventually get recruited in the dividing pool, albeit at different times. On the other hand, the process referred to as activation bias will have a definite impact on the final diversity of the repertoire as cells of lower avidity are ultimately excluded from the dividing pool. Hence, the relative contribution of each phenomenon will determine the quality of the repertoire generated.

Results from the literature do suggest that a limiting display of ligand can significantly influence the course of an immune response possibly by decreasing both the frequency and the diversity of clones recruited in the immune response, as exemplified in this paper (15,61-66). Similarly, others have proposed that the survival of a functionally diverse set of T cells might also be limited by clonal exhaustion to persistently supraoptimal levels of antigen exposure (67,68). Furthermore, *in vivo* and *in vitro* studies have demonstrated that T cells stimulated with excessive doses of ligand show impaired functional responses and increased propensity to undergo apoptosis (28,63,64,69-71). This apparent inability of the immune system to either establish or maintain a diversified repertoire outside a relatively narrow range of ligand concentrations may have important functional implications for a rational design of vaccination strategies. In some instances, the structural diversity necessary for induction of a protective immunity may critically depend on the capacity of the delivery vector employed and/or the antigen processing machinery to set the density of epitopes within a particular window (72,73). What is the importance of selecting a structurally diverse repertoire? It may be to the benefit of the immune system to present a repertoire with the broadest spectrum of affinities to a particular epitope (74). The high avidity T cells which can be triggered by very low amounts of antigen, may not be able to persist under conditions of excessive antigen load (69,71,75). On the other hand, dilution of a protective "high affinity" T cell response by less potent, "low affinity" T cells may adversely impact on the total potency of the

response and represent a costly trade-off to a broader repertoire diversity (76). Additional studies should allow better understanding of the importance of T cell repertoire affinity and diversity in the context of host-pathogen interactions.

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LEGENDS TO FIGURES

FIGURE 1. Influence of TCR ligand dose on the upregulation of activation markers **(A)** The expression of CD25 and CD69 by CD4+ T cells was determined after 48 hrs of stimulation with the indicated doses of SEA. **(B)** Human T cells were stimulated with an optimal dose of 1 ng/ml SEA and a representative experiment (similar data obtained in at least four different blood donors tested) shows the expression of CD69 (mean fluorescence value, plain squares) within a large panel of TCRVβ families covering about 60 % of the CD4+ T cell repertoire, as determined 24 hrs post-stimulation. The percentage of each Vβ-families within the population of CD4+ cells is also indicated (open circles). **(C)** The expression of CD69 was determined at 0.01 pg/ml (left panel) and 0.5 pg/ml (right panel) SEA for different TCRVβ, when gating on CD4+ T cells. The data was obtained after 48 hrs of stimulation, corresponding to the time of maximal CD69 expression previously determined for these two SEA concentrations. Similar data obtained in at least four different blood donors tested.

FIGURE 2. Influence of TCR ligand dose on the timing and proportion of precursors entering the dividing pool. **(A)** Representative dose-response curve of SEA-induced T cell proliferation using human PBMCs (^{3}H-thymidine incorporation after 3 days). **(B)** CFSE-stained cells were stimulated with different concentrations of SEA or PHA (1 μg/ml). Division (CFSE) and expression of the IL-2 receptor α-chain (PE-conjugated anti-CD25 antibody) was measured within CD4+ T cells (PerCP-conjugated anti-CD4) at intervals of 12 hrs for a period of 5 days, at each of the concentrations. **(C)** From the data obtained in **(B)**, an average division number (ADN), representing the average number of divisions that each cell underwent at this specific time, was calculated (see materials and methods) and the results were plotted over time for each SEA concentration.

FIGURE 3. A comparison of the effect of ligand avidity on the kinetics of cell division of different Vβ families. **(A)** CFSE-stained human PBMCs were stimulated with increasing doses of SEA, up to the optimal 1 ng/ml dose. Cells were analyzed by flow cytometry at intervals of 12 hrs for a period of up to 10 days in order to compare the kinetics of cell division between two different TCRVβ families: TCRVβ1 and TCRVβ22

(gated on CD4+ T cells). Representative dot plots are shown at time points day 4.0, 5.0 and 5.5. The events were analyzed on CD4+ T cells whereas the histograms (day 5.0) show the corresponding CFSE profile when gating on CD4+/Vβ+ cells. **(B)** The kinetics of cell division in different SEA-responsive TCRVβ cells was followed as described in figure 4 and for each conditions, the time when (sampling at 12 hrs intervals) a majority (> 50 %) of CD4+/TCRVβ T cells had gone through their first division event was determined in order to define the timing of recruitment of these cells in the dividing population. White squares (no division) indicate that no significant division could be detected after up to 10 days in culture. The results were consistent in kinetics performed with T cells purified from at least 3 different blood donors.

FIGURE 4. Comparison of IL-2 secretion and TCR down-regulation in low vs. high threshold SEA-responsive Vβ TCRs. (A) CFSE-stained human PBMCs were stimulated with 1 ng/ml SEA, or PHA. The progression of different TCRVβ cells into division is represented here after 4 days. The results were gated on CD4+ T cells. **(B)** PBMCs were stimulated with indicated doses of SEA and intracellular IL-2 production was determined within CD4+ Vβ+ T cells (see Materials and Methods).

FIGURE 5. Comparison of ligand-induced TCR down-regulation in low vs. high threshold SEA-responsive TCRVβs. Human T cells were stimulated for 4 hours with different concentrations of SEA and the level of TCR expression was determined in CD4+ cells (anti-CD4 PE-conjugated) using Vβ-specific (FITC-conjugated) antibodies. A representative experiment, from three different blood donors, is shown here. The percentage of TCR expression is compared to simultaneous levels of Vβ expression measured in unstimulated cells. In all TCRVβ tested, the levels of TCR expression dropped synchronously within the first one or two hours (varying between experiments) after which it reached a plateau and remained stable for at least 24 hrs. TCR internalization was totally dependent on the presence of LG-2 cells and on the formation of T cell:LG-2 conjugates (data not shown).

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Figure 1

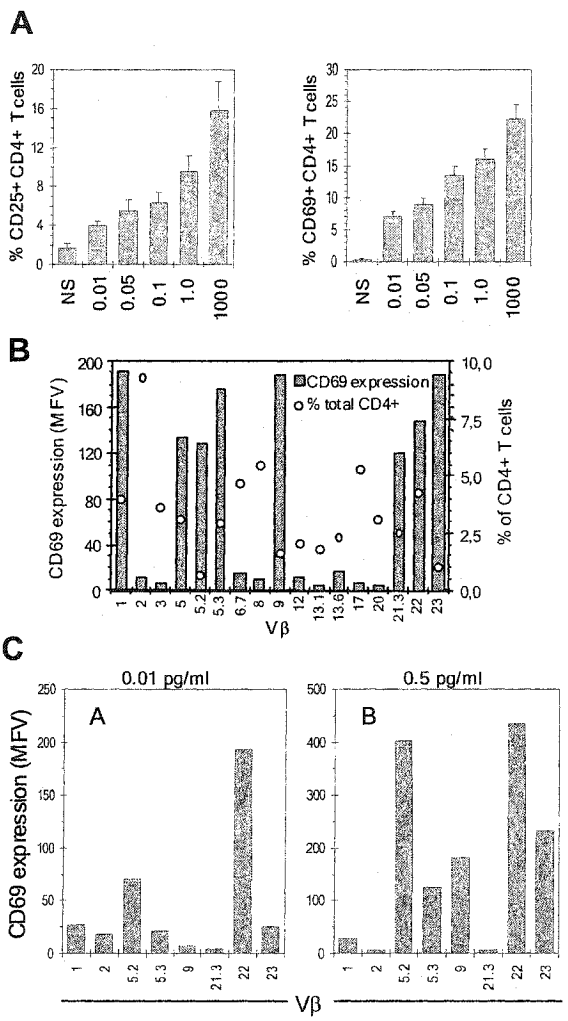

A
% CD25+ CD4+ T cells
% CD69+ CD4+ T cells
NS
0.01
0.05
0.1
1.0
1000
B
CD69 expression (MFV)
% of CD4+ T cells
CD69 expression
% total CD4+
Vβ
C
0.01 pg/ml
0.5 pg/ml
A
B
CD69 expression (MFV)
Vβ

Figure 2

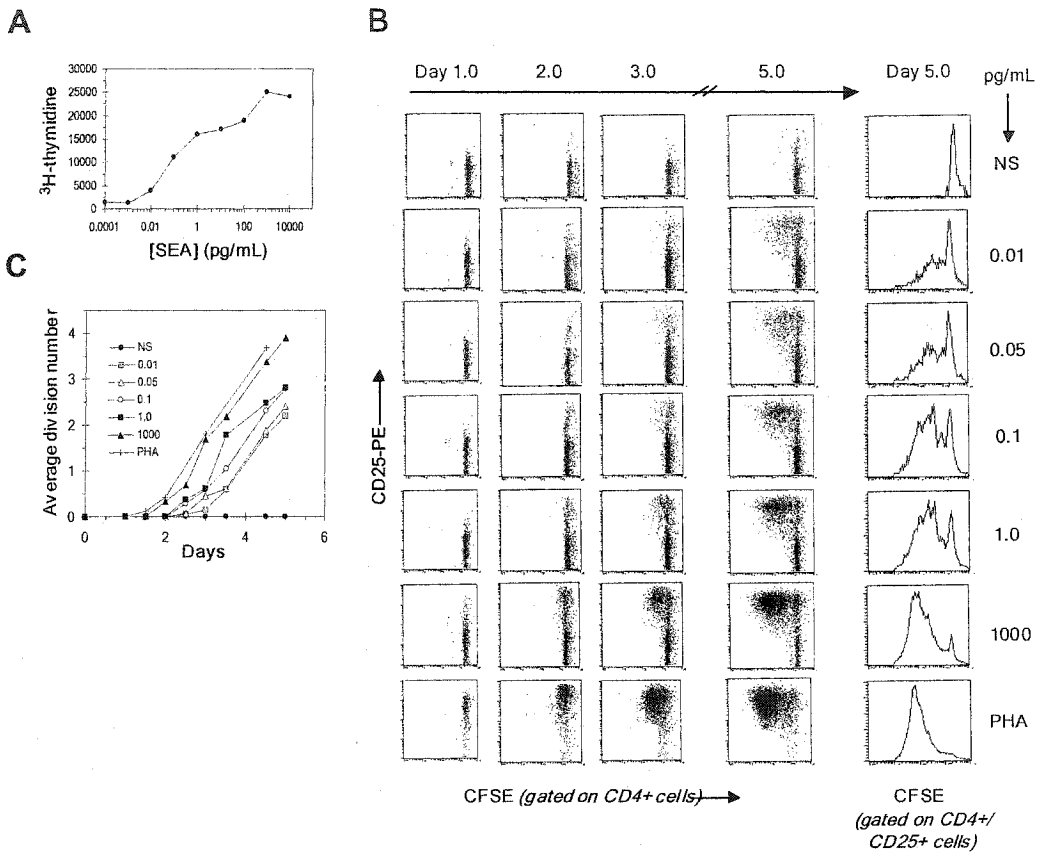
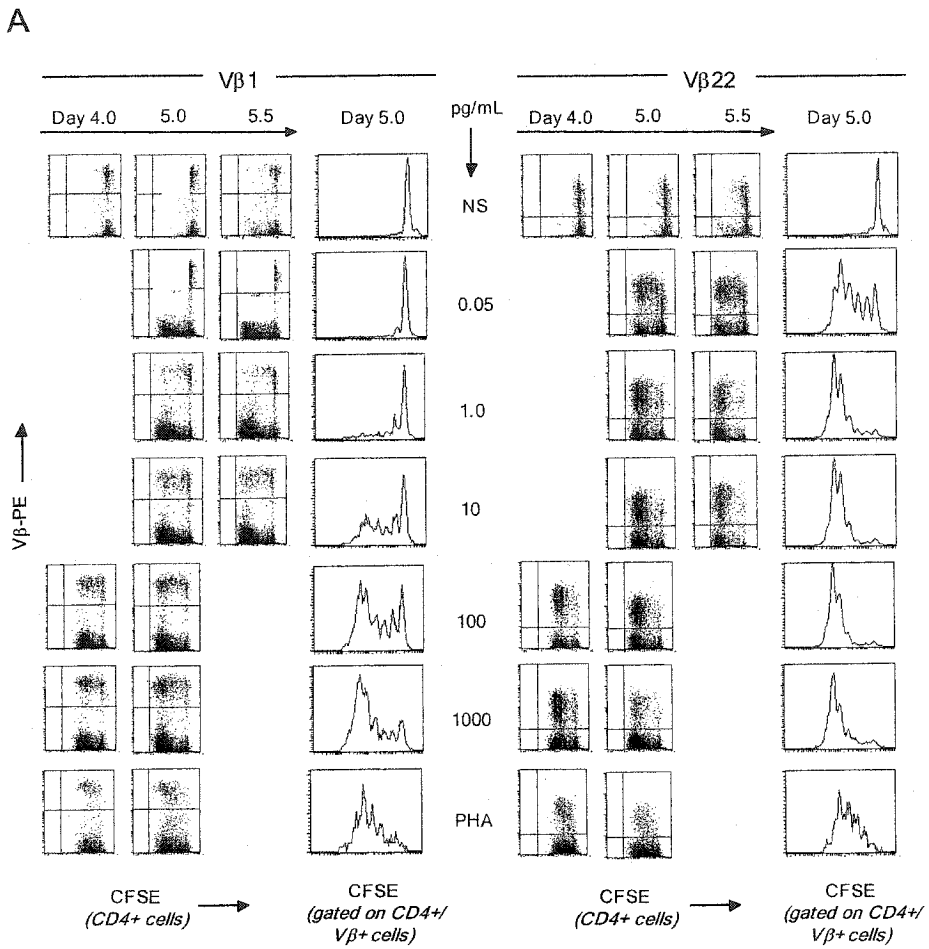
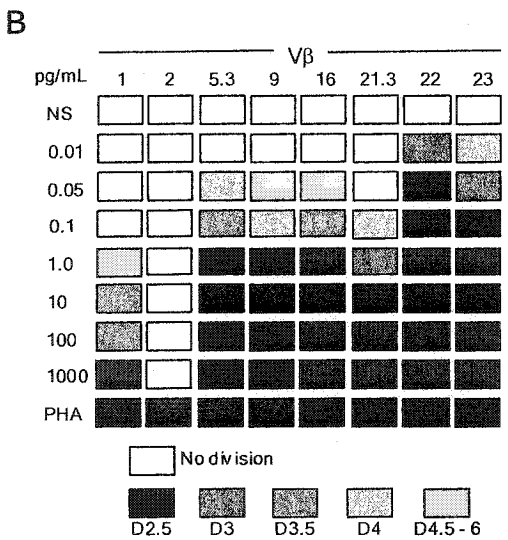

A
^{3}H-thymidine
30000
25000
20000
15000
10000
5000
0
0.0001
0.01
1
100
10000
[SEA] (pg/mL)
C
Average division number
NS
0.01
0.05
0.1
1.0
1000
PHA
0
1
2
3
4
0
2
4
6
Days
B
Day 1.0
2.0
3.0
5.0
Day 5.0
pg/mL
NS
0.01
0.05
0.1
1.0
1000
PHA
CD25-PE
CFSE (gated on CD4+ cells)
CFSE
(gated on CD4+/
CD25+ cells)

Figure 3


A
Vβ1
Vβ22
Day 4.0
5.0
5.5
Day 5.0
pg/mL
NS
0.05
1.0
10
100
1000
PHA
Vβ-PE
CFSE
(CD4+ cells)
CFSE
(gated on CD4+/
Vβ+ cells)


B
Vβ
pg/mL
1
2
5.3
9
16
21.3
22
23
NS
0.01
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10
100
1000
PHA
No division
D2.5
D3
D3.5
D4
D4.5 - 6

Figure 4

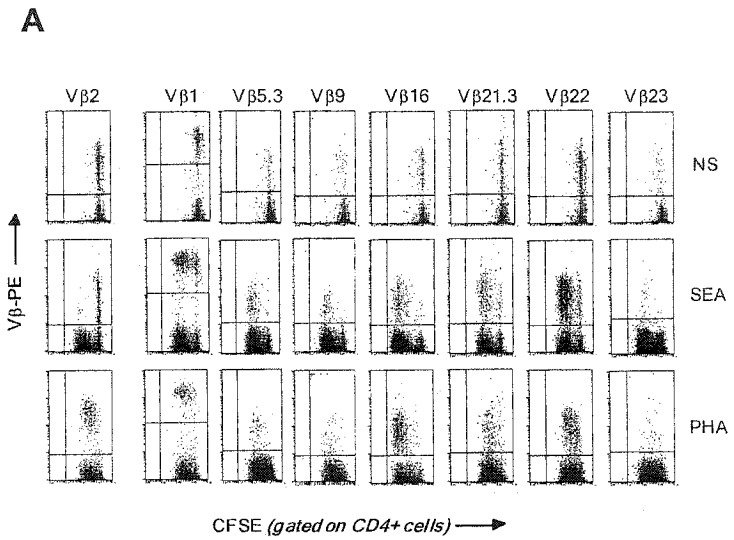
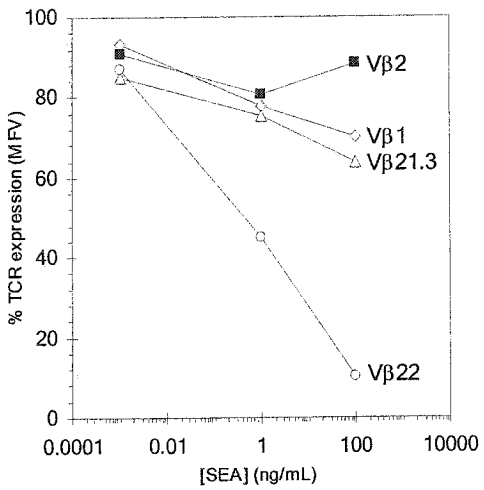

A
Vβ2
Vβ1
Vβ5.3
Vβ9
Vβ16
Vβ21.3
Vβ22
Vβ23
NS
SEA
PHA
Vβ-PE
CFSE (gated on CD4+ cells)
B
Vβ1+ T cells
Vβ2+ T cells
Vβ5.3+ T cells
Vβ21.3+ T cells
Vβ22+ T cells
% of IL-2+ cells
0
2
4
6
8
10
12
14
16
*
0
10
100
1000
[SEA] (pg/mL)

Figure 5


100
80
60
40
20
0
% TCR expression (MFV)
0.0001
0.01
1
100
10000
[SEA] (ng/mL)
Vβ2
Vβ1
Vβ21.3
Vβ22

3 Functional heterogeneity of the human CD4 memory T cell subsets

The study presented in Chapter 2 highlights the importance of the quality of the TCR:ligand interaction in determining the functional outcome of T cell activation. Using an *in vitro* model, we have been able to follow the most proximal events of the expansion of specific TCR clonotypes. This study reveals that the dose of stimulating ligand greatly influences the diversity as well as the kinetics of expansion of the responding T cell population. Moreover, this study also highlights the functional heterogeneity of the CD4 T cell clones undergoing ligand-mediated expansion. However, it is not clear if the heterogeneity observed during the effector phase of the immune response is reflected in the memory T cell population generated.

Work performed by Sallusto and colleagues evidenced that the expression of C-C chemokine receptor 7 (CCR7), which confers a cell with chemotactic response to the "homeostatic" chemokines CCL19 and CCL21, distinguishes two subsets of memory CD4 T lymphocytes that possess differential tissue homing characteristics and effector functions (482). First, "central memory T cells" (T_{CM}) home preferentially to lymphoid organs through expression of CCR7 and CD62L; furthermore, they display little immediate effector function. In contrast, "effector memory T cells" (T_{EM}) express tissue homing receptors associated with inflammation, do not express CCR7, and display more ready-effector function.

With this in mind, we set out to perform a detailed study of the functional properties of human memory CD4 T lymphocyte subsets as defined by the differential expression of CD45RA and CCR7. Using superantigens as polyclonal T cell stimulators, we compared the responsiveness, the proliferative capacity and the ability to express effector molecules of the different memory CD4 T cell subsets. This study allowed us to characterize a new population of effector memory CD4 T cells that was not identified in the original report of Sallusto *et al.* We also demonstrated the existence of functionally heterogeneous subpopulations of T cells within the phenotypically-defined memory subsets.

FUNCTIONAL HETEROGENEITY OF THE HUMAN CD4 MEMORY T CELL SUBSETS[1]

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Key words: Human, T Lymphocytes, memory, cellular differentiation.

INTRODUCTION

The central event in the CD4 T cell-mediated immune response is the recognition, by the T cell receptor (TCR), of ligands presented by self major histocompatibilty complex (MHC) molecules at the surface of antigen-presenting cells (APCs). Following this interaction, a stable adhesive junction involving membrane and cytoskeleton rearrangement is formed between the T cell and the APC (known as the immunological synapse), initiating a cascade of intracellular signals and ultimately leading to complete T cell activation and maturation (1,2).

The T cell response following infection or immunization can be divided into three interrelated phases. First, antigen-specific T cells undergo a burst of clonal expansion during which they acquire effector functions such as cytotoxicity and cytokine secretion. Following this massive expansion, a large proportion of the effector cells (90-95%) are eliminated by activation-induced cell death (AICD). The final outcome of the immune response is the generation and the persistence of long-lived memory T cells in the host (3). Two major subsets of memory T lymphocytes possessing differential effector functions and tissue homing properties have been identified in humans and termed "central memory" (T_{CM}) and "effector memory" (T_{EM}) T cells (4). While T_{CM}, through their expression of the chemokine receptor CCR7 and CD62L, home preferentially to lymphoid organs and display little immediate effector function, T_{EM} cells express tissue homing receptors associated with inflammation and display more ready-effector function (4,5). In CD8 T cells, a third subset of effector cells, characterized by the expression of CD45RA and the lack of CCR7 expression has been identified and termed "terminally differentiated" effector cells or CD45RA+ effector memory cells (T_{EMRA}) (4-6). Similarly, two populations of CD4 memory T cells having different homing and functional properties were found to persist in mice following immunization (7): one small population homing preferentially to lymph nodes and producing IL-2 and a larger population residing in non-lymphoid tissues and producing the anti-microbial cytokine IFN-γ as well as reduced levels of IL-2.

A pathway of T cell differentiation has been established in CD8 T cells whereby, following antigen encounter, T cells proliferate and gain effector properties while undergoing phenotypic changes that modify their tissue homing properties (8,9). Antigen-specific cells are recruited into a pre-memory/effector subset possessing a naïve-like phenotype (CD45RA+ CCR7+) before reaching the T_{CM} and T_{EM} maturation stages (CD45RA- CCR7+ and CD45RA- CCR7-, respectively). Ultimately, a stage characterized by strong effector functions but weak proliferative capacity has been identified in CD8 T cells (CD45RA+ CCR7-) (5). A refinement of this model, based on the expression of co-stimulatory molecules CD27 and CD28, was recently proposed (10). The gain in effector functions, as defined by IFN-γ expression as well as presence of perforin and granzyme B-containing granules, would be correlated with the loss of these co-stimulatory molecules at the surface of CD8 T cells.

In this paper, we characterize the phenotypic and functional properties of the different human CD4 T cell subsets as defined by CD45RA and CCR7 expression. First, we identify in human peripheral blood a subset of CD4 T cells expressing CD45RA but lacking CCR7 expression that displays strong effector functions, as measured by IFN-γ and perforin expression, but expressing markers associated with replicative senescence. Moreover, we show that the naïve, memory and effector/memory CD4 T cell subsets differ in their responsiveness to TCR stimulation and also by the array of cytokines they produce.

METHODS

Flow cytometry

The fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (M-A251), anti-CD69 (Leu-23), anti-CD45RA (HI100), anti-perforin (δG9), anti-IFN-γ (25723.11), anti-IL-2 (MQ1-17H12), the phycoerythrin (PE)-conjugated anti-CCR7 (3D12), CD27 (M-T271), the peridinin chlorophyll-*a* protein (PerCP)-conjugated anti-CD4 (Leu-3a) and the allophycocyanin (APC)-conjugated anti-CD45RA (HI100) and CD3 (UCHT1) were purchased from BD Biosciences (San Jose, CA). The purified anti-CD27 (1A4CD27) was purchased from Beckman Coulter (Miami, FL); the Tri-Color-conjugated goat anti-mouse IgG secondary antibody was purchased from Caltag Laboratories (Burlingame, CA). The KLRG1 antibody was generated as previously described (11). Data acquisition was performed with a FACS Calibur or LSR II (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Intracellular staining

Intracellular cytokines were measured after 6 to 8 hours of stimulation using procedures previously described (12). Briefly, PBMCs ($2x10^6$/ml) were incubated in RPMI 1640 media (Gibco BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS) with either no stimulus, PMA (50 ng/ml) and Ionomycin (500 ng/ml) or a mix of superantigens for 6 hours. Brefeldin A (10 ug/ml) was added after 90 minutes of stimulation. After stimulation, cells were stained for extracellular markers, fixed, permeabilized and stained with monoclonal antibodies against IL-2 and/or IFN-γ. Intracellular perforin staining was performed directly *ex vivo* without any *in vitro* stimulation.

CD4+ T cell purification and sorting

PBMCs from healthy blood donors were isolated using Ficoll-Hypaque density gradient centrifugation. CD4+ T cells were purified from total PBMCs by negative selection using CD4+ T cell isolation kit combined with autoMACS magnetic sorter (Miltenyi Biotec, Auburn, CA). Following purification, CD4+ T cells were highly enriched (>97%) as

determined by flow cytometry. Purified CD4+ T cells were stained for CD45RA and CCR7 and the four different populations were sorted using a MoFlo Cell Sorter (DakoCytomation, Fort Collins, CO). Following sorting, each population was highly enriched (>95%) as measured by flow cytometry. For experiments involving *in vitro* stimulation, PBMCs from the same blood donor were stained using PE-conjugated anti-TCR and the TCR-negative cells were sorted and used as antigen- presenting cells.

CFSE and PKH26 labeling

Cells were resuspended in phosphate buffered saline (PBS) at a density of $10x10^6$/ml. 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) was added to a final concentration of 0.5 μM and cells were incubated in the dark for 8 minutes at room temperature. Labeling reaction was stopped by adding two volumes of FCS and CFSE-labeled cells were washed 3 times with PBS supplemented with 10% FCS prior to use. PKH26 (Sigma-Aldrich, St-Louis, MO) labeling was performed according to the manufacturer's protocol.

T cell stimulation

Cells were cultured in RPMI 10% FCS at a concentration of $2x10^6$/ml. Polyclonal T cell stimulation was done using a mix of superantigens (SEA, SEB, SEC_3, SED, SEE, TSST-1, SpeC) at a final concentration of 1 to 100 ng/ml. Highly purified SEA, SEB, SED, SEE and TSST-1 were purchased from Toxin Technologies (Sarasota, FL). Recombinant SEC_3 and SpeC were a generous gift from Dr. Roy Mariuzza. For experiments using sorted cells, purified CD4+ T cells were cultured with antigen-presenting cells at a 2:1 ratio.

RESULTS AND DISCUSSION

The CD4 T cell population comprises four distinct subsets as assessed by CD45RA and CCR7 expression. The initial study aiming at identifying subsets of memory T cells based on CD45RA and CCR7 expression revealed the existence of three subsets of CD4 T cells and four subsets of CD8 T cells in healthy individuals (4). However, by screening several blood donors, we found that some healthy individuals have a small population (< 5% of total CD4 T cells) of CD4+ T cells expressing CD45RA but lacking CCR7 expression (figure 1). By analogy to CD8 T cells (6), we defined this population as CD45RA+ effector memory cells (T_{EMRA}).

Effector memory CD4 T cells show increased functional sensitivity to TCR stimulation. It is well described that memory T cells are functionally more responsive than naïve T cells (13). They acquire effector properties, such as cytokine secretion and cytotoxicity, more rapidly than naïve cells. However, it is not known whether there are differences in the activation threshold of central, effector memory and CD45RA+ effector memory CD4 T cells. In mouse CD8 T cells, Kersh et al. (14) observed that T_{CM} are more responsive than T_{EM} to antigen upon secondary infection due to their increased signaling capacity. The responsivess of the CD4 subpopulations to TCR stimulation was examined by measuring the up-regulation of the early activations markers CD69 and CD25 following activation with different doses of superantigens. We used superantigens (instead of anti-CD3 or anti-TCR antibodies) for polyclonal T cell activation because their intrinsic properties, such as their affinity for the TCR (15,16) as well as their ability to induce the formation of the immunological synapse (17), closely mimics those of typical antigenic peptides. As shown in Figure 2, CD45RA-CCR7- effector memory CD4 T cells are activated at lower concentrations of superantigens as compared to the naïve, T_{CM} and T_{EMRA} subpopulations. CD45RA-CCR7- CD4 T cells consistently (n=4) up-regulated CD25 and CD69 at ligand concentrations about 100 times lower than CD45RA+CCR7+ and about 10 times lower than CD45RA-CCR7+ and CD45RA+CCR7-. Differences in responsiveness of the subsets were not due to difference in TCR or CD3 expression levels (data not shown).

The different memory subsets display distinct cytokine secretion profiles. Using intracellular cytokine staining, we next wanted to compare the capacity of each subset to produce cytokines following brief stimulation with a mix of superantigens. As shown in Fig. 3A, while the T_{CM} subpopulation contains mostly IL-2-producing cells and the T_{EMRA} subset contains exclusively IFN-γ-secreting cells, both cytokines are secreted by CD4 T cells from the T_{EM} subset. In some experiments, a small proportion of naïve T cells produced IL-2 at the highest dose of superantigens. Similar results were obtained when cells were stimulated with PMA/ionomycin (except naïve CD4 T cells that produced IL-2, as previously reported (4)), indicating that the differences in cytokine secretion profiles were not due to differences in TCR responsiveness but rather to intrinsic cytokine secretion capacity of each memory subset (data not shown).

Since the previous staining for IFN-γ and IL-2 were not performed in the same test tube, we were not able to discriminate between two non-mutually exclusive possibilities: It is possible that the CD45RA-CCR7- population is homogenous in cells producing both cytokines simultaneously or maybe this population is heterogeneous and comprises cells producing exclusively IL-2 and other cells secreting only IFN-γ. To verify these two possibilities, we performed five-color stainings to simultaneously detect IFN-γ- and IL-2-secreting cells in the effector memory population (Fig. 3B). The T_{EM} subset appears to contain three distinct CD4 T cell subpopulations that can be distinguished by the expression of IL-2 only, IFN-γ only or both cytokines. It can be hypothesized that the differential expression of these cytokines identifies subsets of CD4 memory T cells at different stages of differentiation along a linear differentiation pathway. These results strongly suggest that the phenotypic categorization of T cells into subsets solely based on the expression of a combination of cell-surface markers can mask the existence of subpopulations having distinct functional properties but expressing a common set of markers.

The CD45RA+CCR7- subset is enriched in CD4 T cells containing perforin granules and expressing markers associated with replicative senescence. It is now well

described that some CD4 T cells, known as "CD4+ CTLs", can express perforin and mediate cytotoxic killing directly *ex vivo* (18,19). These cells are usually found at low levels in healthy individuals but are markedly expanded at all stages of chronic viral infections (19). In CD8 T cells, it has been shown that the terminally differentiated T_{EMRA} subset is enriched in cells expressing high perforin levels (4,5). To verify if a particular subset of CD4 T cells is enriched in CD4+ CTLs, we performed direct *ex vivo* intracellular staining to measure perforin expression in these subsets. We observed that a high proportion of CD45RA+CCR7- CD4 T cells (usually between 20 and 40%) possess intracellular stocks of perforin granules (Fig. 4A). Moreover, a significant fraction (between 5 to 10%) of effector memory (CD45RA-CCR7-) CD4 T cells also express perforin. As shown in Fig 4B, perforin expression in CD4 T cells is restricted to a subpopulation that has down-regulated the expression of CD27, similar to the observations made in CD8 T cells (10,20). Since the majority of CD27- cells are found in the CCR7- subsets (either CD45RA+ or CD45RA-), we can deduce that CD4 CTLs are mostly restricted to the CD45RA+CCR7-CD27- and CD45RA-CCR7-CD27- subsets. CD8 T cells having this phenotype have been characterized as highly differentiated effector memory T cells (10,21). Therefore, it appears that CD4 T cells reaching a highly differentiated phenotype begin to express cytotoxic molecules. It is unclear, however, if these CD4 T cells play a specific role during the immune response (by killing antigen-bearing MHC class II-positive cells) or if the expression of perforin by CD4 T cells is "abnormal" (or unspecific) and results from their highly differentiated state.

It has been demonstrated in various experimental models that T cells can express receptors usually expressed by subsets of Natural Killer (NK) cells. Recently, several groups showed that subsets of effector/memory CD4 and CD8 T cells were highly enriched in cells expressing killer cell lectinlike receptor G1 (KLRG1), an inhibitory cell surface receptor initially found on a mast cell line and on a subpopulation of NK cells (11,22,23). The expression of this receptor on T cells requires TCR engagement (24) and it has been strongly linked with impairment in proliferative capacity (11,25). In mice, expression of this receptor by T cells appears to be associated with a history of extensive cell division (25). Consistent with previously published data, we found that KLRG1 was

highly expressed on a large fraction (usually between 50 to 90%) of CD45RA+CCR7- CD4 T cells and at lower levels on a significant fraction (about 30%) of effector memory CD45RA-CCR7- cells (Fig. 4C). We confirmed that CD4 T cells expressing KLRG1 are severely impaired in their ability to proliferate following activation with superantigens (Fig. 4D). We obtained similar results by stimulating sorted KLRG1- and KLRG1+ CD4T cells with superantigen-loaded autologous APCs (data not shown), confirming that the results obtained in Fig. 4D were really due to an inability of KLRG1+ T cells to proliferate and not caused by an activation-induced down-regulation of KLRG1 on proliferating cells. Moreover, comparison of IFN-γ secretion by KLRG1+ and KLRG1- cells revealed that T cells expressing KLRG1 are highly enriched in IFN-γ-producing cells (Fig. 4E). Typically, we found that 20-30% of KLRG1+ cells secreted IFN-γ as compared to 1-5% for KLRG1- cells. Similar to what has been described in CD8 T cells (4,5), the T_{EMRA} subset in CD4 T cell is highly enriched in cells having strong effector function, possessing cytotoxic potential but having impaired proliferative capacity.

Taken together, the data presented in this paper expand the previously characterized heterogeneity of the memory CD4 T cell population (4). Although found at much lower level as compared to CD8 T cells, a subset of CD4 T cells having a CD45RA+CCR7- phenotype was characterized. We demonstrate that this subset is enriched in CD4 T cells having strong direct *ex vivo* IFN-γ secretion ability but impaired in their capacity to produce IL-2 and to proliferate following polyclonal stimulation with superantigens. This subset also contains a significant proportion of CD4 T cells with cytotoxic potential (known as CD4+ CTLs), as shown by their expression of perforin-containing granules. Moreover, we show that the effector memory (CD45RA-CCR7-, T_{EM}) CD4 T cell subset is not homogenous but comprises subpopulation of cells having distinct functional properties, as demonstrated by their differential expression of IL-2 and IFN-γ. Additional studies should allow a better understanding of the mechanisms underlying the differentiation and maintenance of these memory CD4 T cell subpopulations as well as their precise role for immune protection.

LEGENDS TO FIGURES

Figure 1. A CD4 T cell subset having a CD45RA+CCR7- phenotype is found in a small fraction of healthy individuals. CD4+ lymphocytes from healthy blood donors were stained with monoclonal antibodies to CD45RA and CCR7. Numbers indicate the percentage of CD4+ lymphocytes in each quadrant. Representative results are shown.

Figure 2. CD45RA-CCR7- effector memory CD4 T cells show enhanced responsiveness to superantigen stimulation. PBMCs were stimulated with different concentrations of superantigens and the expression of CD69 (A) and CD25 (B) in the different subsets was measured after 12 to 24 hours of stimulation. The distribution of CD4+ T cells among the different subsets remains unchanged during the time of stimulation (data not shown).

Figure 3. Immediate cytokine secretion by the different CD4+ T cell subsets. PBMCs were stimulated with different concentrations of superantigens for 6 to 8 hours. Brefeldin A was added after 90 minutes of stimulation. Following stimulation, PBMCs were stained with monoclonal antibodies to CD4, CD45RA and CCR7, fixed, permeabilized and stained with antibodies to IL-2 or IFN-γ. A. The percentage of IL-2- and IFN-γ-producing cells in each subset is represented in grey and black, respectively. B. Simultaneous intracellular detection of IFN-γ and IL-2 in CD4+CD45RA-CCR7- lymphocytes was performed using 5-color flow cytometry. Results are representative of three independent experiments.

Figure 4. CD45RA+CCR7- CD4 T cells represents a subset having strong effector function but limited proliferative capacity. Staining for intracellular perforin and differentiation markers CD45RA and CCR7 (A) and CD45RA and CD27 (B) was conducted directly *ex vivo* using fresh or cryopreserved PBMCs. KLRG1 (C) or KLRG1 and IFN-γ (E) expression by the different CD4+ T cell subsets. D. T cell proliferation was assessed by labeling the PBMCs with PKH26, a lipophilic cell tracker dye and by staining with CD4-APC and KLRG1-Alexa 488 antibodies following 96 hours of stimulation with superantigens. Dashed gray lines represent isotype controls. Representative results of at least three independent experiments are shown.

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Figure 1

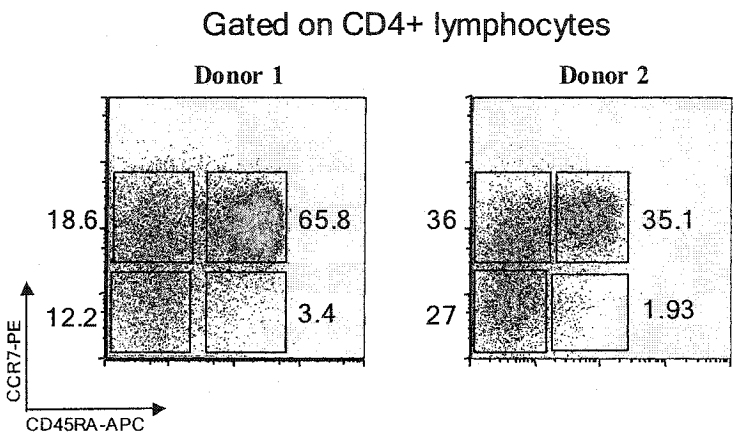
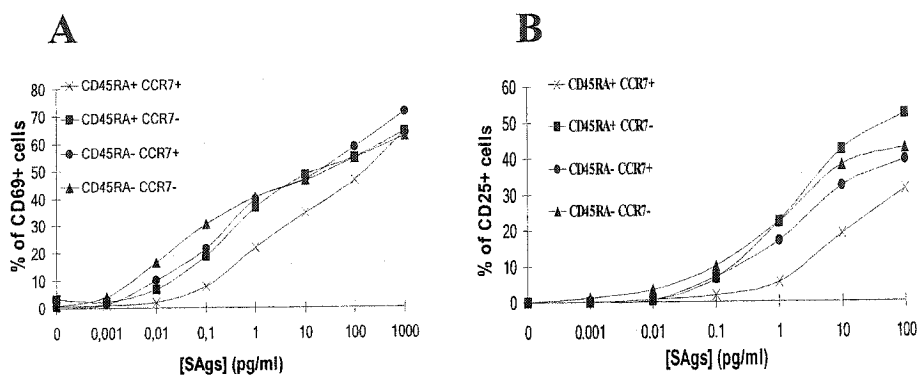

Gated on CD4+ lymphocytes
Donor 1
Donor 2
18.6
65.8
12.2
3.4
36
35.1
27
1.93
CCR7-PE
CD45RA-APC

Figure 2


A
B
CD45RA+ CCR7+
CD45RA+ CCR7-
CD45RA- CCR7+
CD45RA- CCR7-
% of CD69+ cells
% of CD25+ cells
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[SAgs] (pg/ml)

Figure 3

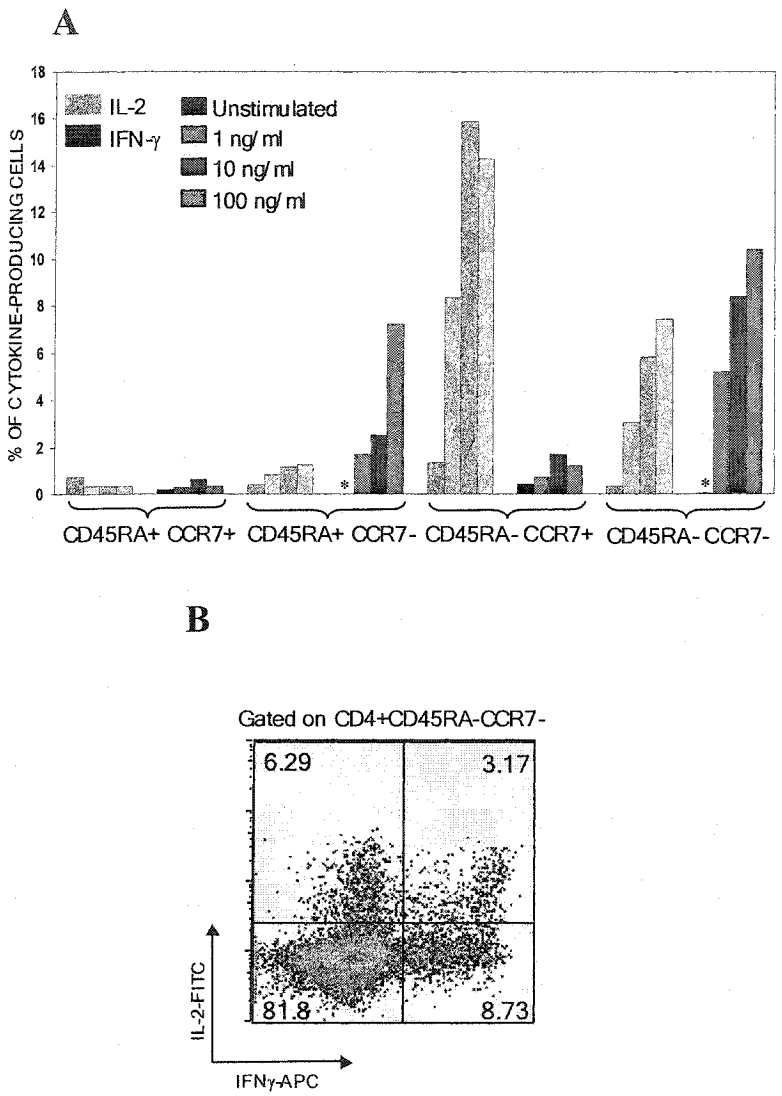
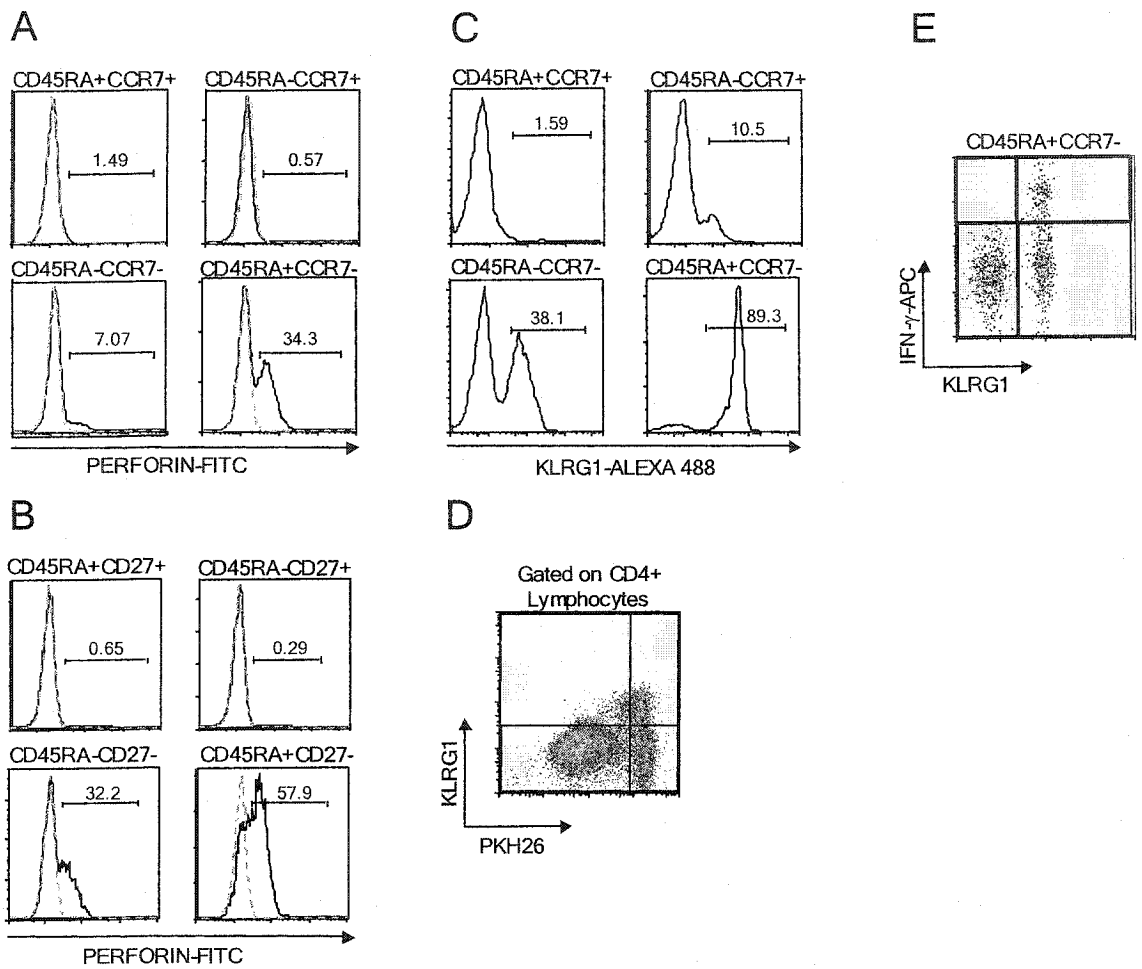

A
% OF CYTOKINE-PRODUCING CELLS
IL-2
IFN-γ
Unstimulated
1 ng/ml
10 ng/ml
100 ng/ml
CD45RA+ CCR7+
CD45RA+ CCR7-
CD45RA- CCR7+
CD45RA- CCR7-
B
Gated on CD4+CD45RA-CCR7-
6.29
3.17
81.8
8.73
IL-2-FITC
IFNγ-APC

Figure 4


A
CD45RA+CCR7+
1.49
CD45RA-CCR7+
0.57
CD45RA-CCR7-
7.07
CD45RA+CCR7-
34.3
PERFORIN-FITC
B
CD45RA+CD27+
0.65
CD45RA-CD27+
0.29
CD45RA-CD27-
32.2
CD45RA+CD27-
57.9
PERFORIN-FITC
C
CD45RA+CCR7+
1.59
CD45RA-CCR7+
10.5
CD45RA-CCR7-
38.1
CD45RA+CCR7-
89.3
KLRG1-ALEXA 488
D
Gated on CD4+
Lymphocytes
KLRG1
PKH26
E
CD45RA+CCR7-
IFN-γ-APC
KLRG1

4 Original contribution to scientific knowledge

The results presented in this thesis contribute to scientific knowledge in several aspects.

The work presented in Chapter 2 represents a unique *in vitro* model making use of superantigens to study the expansion of the TCR repertoire. Data from chapter 2 allowed the identification of novel SEA-reactive TCRVβ families and provided the first direct demonstration of the existence of a hierarchy in the sensitivity of different TCRVβs for a superantigen. This report directly demonstrated the influence of TCR ligand concentration on the kinetic and diversity of the responding T cell population. Results in chapter 2 also directly established that the onset, but not the rate, of T cell proliferation is dependent on ligand concentration. Moreover, we showed that a clonotypically diverse T cell population that appears homogenous in terms of activation markers upregulation and proliferation display heterogeneity in the ability to secrete cytokines.

The work presented in chapter 3 is an original study of the phenotypical and functional properties of CD4 T cell subsets as identified by CD45RA and CCR7 expression. Critically, a population of $CD45RA^{+}$ $CCR7^{-}$ CD4 T cells with strong effector function and decreased proliferative capacity was characterized for the first time. We showed that this population is enriched in CD4 T cells containing perforin granules (CD4+ CTLs) and in cells expressing markers associated with replicative senescence. This report also demonstrated that the different subsets clearly differ in their sensitivity to TCR engagement and in the panel of cytokines they produce. Finally, data from chapter 3 showed marked heterogeneity, in terms of IFN-γ and IL-2 secretion, in the previously identified effector memory (CD45RA- CCR7-) population.

5 Discussion

5.1 Basis for the TCR Vβ specificity of SEA

As exemplified in chapter 2, SEA stimulates a large but distinct set of T cells based on the expression of specific TCR Vβ domain with apparently little or no contribution from other parts of the TCR normally involved in peptide/MHC recognition (596;636;637). The binding of a single soluble TCR Vβ chain (mouse Vβ3), in the absence of a TCR α chain, to SEA complexed with MHC class II molecules has been reported more than 10 years ago (628). Although the crystal structure of SEA complexed with the TCR has not yet been resolved, different strategies have been used to identify regions, in both SEA and TCR, which are important for their interaction.

SEA and SEE possess a high degree of sequence (83% homology) and structural similarities but demonstrate reciprocal patterns of Vβ-specific T cell activation (see table III) (534;638;639). For example, SEA (but not SEE) stimulates human Vβ5.3- and Vβ9.1-bearing T cells whereas SEE (but not SEA) activates human Vβ5.1 and Vβ8.1-bearing T cells (534;640). These characteristics make them good candidates for functional region mapping. Using SEA-SEE hybrids, four independent groups identified the residues that control the TCR Vβ specificity of these SAgs (534;640-642). These studies revealed a three amino acid dimorphism (200, 206 and 207) between SEA and SEE that is responsible for differential stimulation of T cells (Figure 8). All three dimorphic residues, located in the carboxy-terminal end, play a role in preferential stimulation of one specific TCR Vβ segment versus another. Moreover, three residues near the amino-terminal end of SEE (20, 21 and 24) are also involved in TCR binding and contribute to Vβ specificity (642;643). The theoretical model for the structure of SEE predicts that the amino- and carboxy-terminal Vβ determinants of SEE are found on two structurally adjacent, solvent-exposed loops and are available to contact the TCR (642;644). Interchanging residues 20-24 and 200-207 of SEA for those corresponding in SEE confers the hybrid toxin with the same Vβ specificity and the same level of

superantigenic activity as wild-type SEE, clearly demonstrating that these two regions constitute the TCR-binding site (642;644).

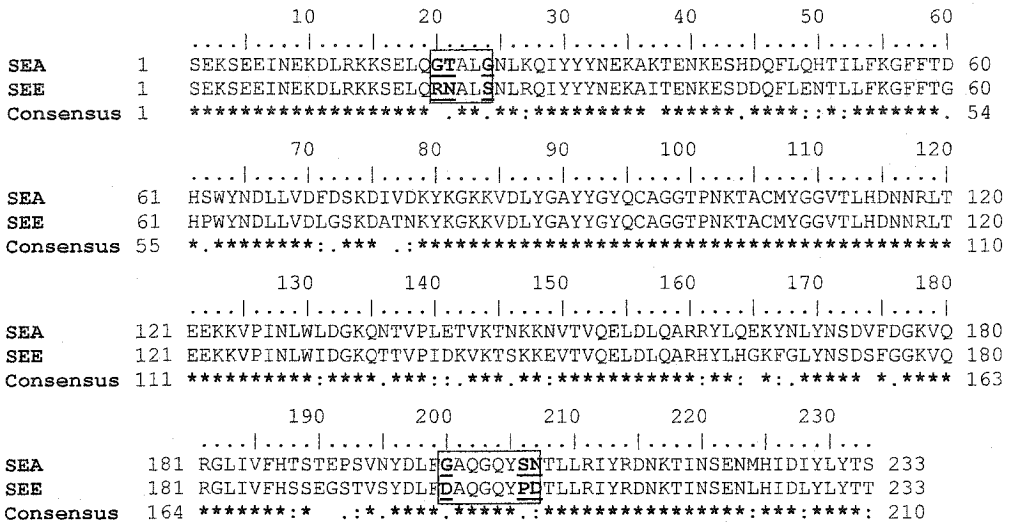


Figure 8. Comparison of the amino acid sequences of SEA and SEE. *The regions important in determining TCR Vβ specificity are boxed and the specific residues involved are in bold and underlined. The sequence alignment has been generated using Clustal X (645)*

To identify the region(s) of the TCR Vβ chain involved in the interaction with SEA, Pontzer et al. generated overlapping synthetic peptides corresponding to regions of the mouse Vβ3 chain (646). One of these peptides, corresponding to residue 57 to 77 from the Vβ3 segment, blocked the binding of the soluble β chain to the MHC-bound SEA and completely inhibited SEA-induced proliferation and IFN-γ production of spleen cells. These data strongly suggest that the region encompassing amino acids 57-77 of Vβ3 is directly implicated in the interaction with SEA.

In order to identify possible TCR Vβ residues involved in the recognition of SEA, an amino acid sequence alignment of human and mouse SEA-responsive and unresponsive Vβ sequences has been generated. This alignment permits the identification of specific features highly conserved among the responsive Vβ families (Figure 9). A conserved tetrapeptide sequence (consensus: X – R (Q) – F – S, where X is a basic amino acid or

asparagine (N)) located in the HV4 region (between residues 60-65) is found in all SEA-responsive Vβs but not in the unresponsive ones. These observations are in agreement with previously published studies that identified this amino acid cluster as a determinant for interaction with SEA and SEE (646;647). Interestingly, Vβ22, which is clearly the most sensitive Vβ in functional experiments, contain a glutamine (Q) at position 2 of the consensus sequence while all the other responsive Vβs have an arginine (R) residue, suggesting that this amino acid plays a role in stabilizing the TCR-SEA interaction and may explain the enhanced reactivity of Vβ22+ T cells.

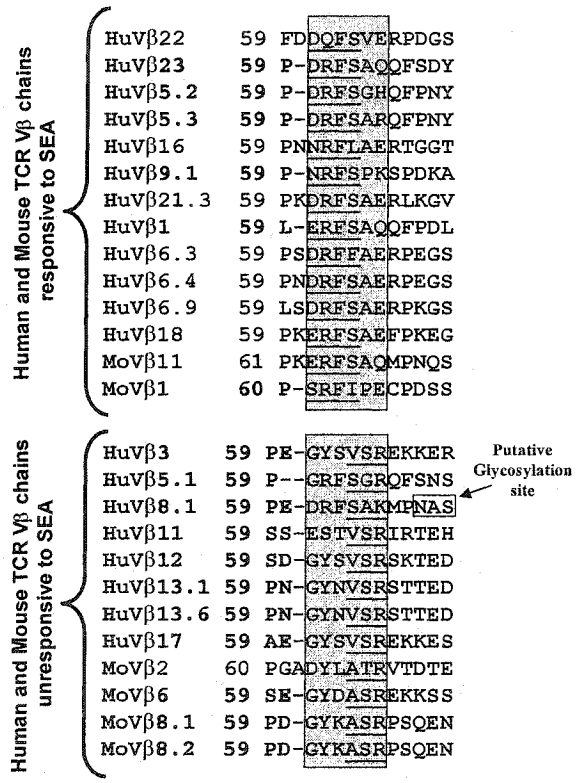


Figure 9. Sequence alignment of the HV4 region of responsive and unresponsive TCR Vβ chains.

Vβ8.1 appears to be an exception to the rule mentioned above. Despite the fact that it contains the consensus sequence, the Vβ8.1 chain does not interact strongly with SEA as deduced from the absence of stimulation of Vβ8.1-bearing T cells in functional assays.

However, the presence of a putative glycosylation site (N-A-S) just after the consensus sequence suggests that the presence of sugar residues in the vicinity of the binding site could possibly affect the SEA-Vβ8.1 interaction. It is also possible and very likely that other regions from the TCR Vβ chain, such as the CDR1 and CDR2 loops, are involved in stabilizing the interaction. Therefore, the amino acids found in those regions in the Vβ8.1 sequence would not permit the stable contact with SEA, explaining the lack of response of Vβ8.1+ T cells following SEA stimulation.

Another interesting observation can be made by analyzing the members of the Vβ5 family. As demonstrated in chapter 2, Vβ5.2+ and Vβ5.3+ T lymphocytes are activated by SEA (but not by SEE) while Vβ5.1+ T cells are stimulated by SEE (but not by SEA) (508). It appears that the small, uncharged glycine (G) residue found at position 1 of the consensus sequence of Vβ5.1 and replaced by a negatively-charged aspartic acid (D) in Vβ5.2 and 5.3 does not allow high-affinity binding to SEA but is favoured in the interaction with SEE. A likely explanation for this differential binding can be deduced by comparing the residues previously shown to confer Vβ specificity of these two toxins (positions 200, 206 and 207, see figure 8). SEE contains two negatively-charged aspartate residues (200 and 207) that could inhibit, by electrostatic repulsion, the binding of Vβ5.2 and 5.3 (which also contain a negatively-charged aspartate) but favour the interaction with the small, uncharged glycine of Vβ5.1. In contrast, SEA contains a small glycine residue at position 200 and the presence of an aspartate in the TCR Vβ domain would be necessary to ensure strong interaction between the two proteins.

5.2 Affinity/avidity of the TCR-MHC/ligand interaction and biological outcomes

It is now generally accepted that T cell fate is greatly influenced by the amount of signal (signal strength) that a T cell received during the interaction with APCs (648). Signal strength refers to the overall amount of signal transduced through the TCR and is determined by four main factors:

1. the density of TCR ligands displayed on the APCs, which influences the rate of TCR triggering;
2. the affinity of the TCR for the ligand, which determines the extent of signal accumulation;
3. the density of APCs, which determines the frequency and duration of T cell stimulation;
4. the concentration and nature of co-stimulatory molecules, which dictates the extent of signal amplification.

Because they have not been studied directly within the context of this thesis, the effect of the density of APCs, as well as the nature and concentration of co-stimulatory molecules will not be further discussed.

5.2.1 The density of TCR ligands influences the T cell response *in vitro*

The data presented in chapter 2 clearly demonstrate that the amount of SEA-MHC class II complexes displayed by the APCs to the T cells qualitatively and quantitatively influence T cell response. We show that decreasing the amount of TCR ligand has three major effects. First, the onset of T cell activation and expansion is significantly delayed (up to 36 hours as compared to an optimal concentration). Second, the number of different T cell clones reaching the activation threshold is diminished, restricting the TCR diversity of the responding population. Third, activated T cells do not exhibit all the effector properties associated with full T cell activation.

5.2.1.1 Why is T cell activation delayed at low ligand concentrations?

This phenomenon can be explained in the context of a temporal summation model for T cell activation (329). The temporal summation mechanism is well characterized in neuronal cell activation (649); the frequency of pre-synaptic signals is translated into the size of a post-synaptic potential. According to this model, T lymphocytes accumulate,

over time, short-lived intracellular signalling intermediates (second messengers) originating from successively triggered TCRs (figure 10). Thus, the brief and weak signals generated by individually triggered TCRs that alone are unable to elicit a response can be summed up over time to reach the threshold for activation. A high TCR ligand density would allow the very rapid accumulation of several short-lived signals that are integrated to a total signal sufficient to reach the activation threshold. However, at low ligand concentrations, the number of triggered TCRs in a given time period are few. Therefore, the time required for accumulation of several individual signals is long, leading to a delayed T cell activation. If the signals are very short-lived, this phenomenon is even more prominent because by the time a second signal is generated, the first one would be almost completely extinct.

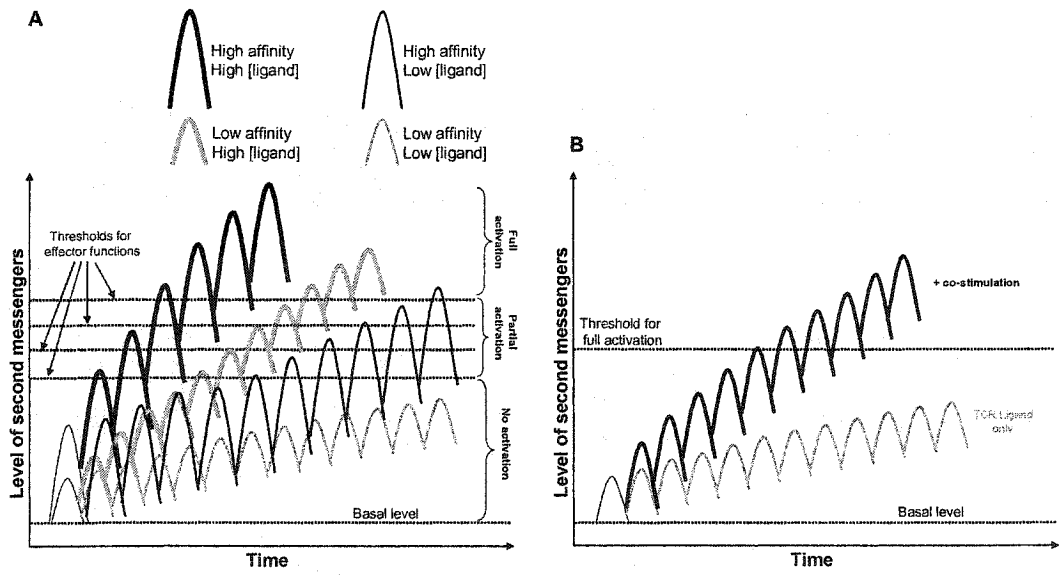


Figure 10. The temporal summation model for T cell activation. *Individual TCR engagement events lead to the transient generation of second messengers (phosphorylated proteins, Ca^{2+}, etc.). The amplitude and the duration of the individual signals are primarily determined by the stability of the TCR-ligand interaction (off-rate) while the frequency of these signals is primarily dictated by the number of available ligands.* ***A)*** *Therefore, the balance between TCR affinity and ligand concentration will determine the outcome of T cell activation.* ***B)*** *Co-stimulation does not increase the number of triggered TCRs but rather enhances the accumulation of second messengers, decreasing the number of TCRs necessary to reach the activation threshold.*

The amplitude and the duration of individual signals would depend on the affinity, particularly the off-rate, of the TCR for its ligand. A high affinity, long-lived TCR-ligand interaction would promote the strong accumulation of second messengers for a prolonged period of time while a low affinity interaction would lead to small and very transient accumulation of second messengers. According to this hypothesis, a high affinity interaction could therefore compensate for a low number of ligands while a high concentration of ligand can compensate for a low affinity binding. This is in agreement with functional results showing an inverse correlation between TCR affinity and the number of ligands (or the number of TCRs) required for T cell activation (278;337;650-652).

Co-receptors and co-stimulatory molecules are believed to lower both the number of TCRs and the signalling time required for a T cell response by extending the time course of an individual signal, thereby facilitating the summation of intermediate signalling events (figure 10B). As a matter of fact, experiments comparing T cell activation in presence or absence of co-stimulatory receptors revealed that co-stimulation lowers the number of TCRs and/or the ligand concentration required to reach the activation threshold and decrease the time of commitment for activation (197;266;328;653-656). Moreover, this model also predicts that co-stimulation will be absolutely necessary for low affinity T cells and at low ligand concentration but dispensable for high affinity T cells and at high antigen doses; results from the literature strongly support this prediction (331;657).

An essential feature of the temporal summation model is that signalling events resulting from triggered TCRs are gradually accumulating over time. Experiments measuring the calcium response in T lymphocytes have revealed that there is a delay between TCR ligation and the onset of the calcium signal (218;336). The duration of this delay correlates with the strength of the activating stimulus; stronger stimuli, defined here by Ag dose, giving a more rapid response. Similar conclusions were reached by Muller *et al.* when evaluating the extent of PTK activation following T cell activation with varying doses of antigen (658). Recently, the incremental accumulation of short-lived intermediates of the extracellular signal-regulated kinase (ERK) family was observed

following T cell stimulation (659). The rate and extent of the accumulation of intermediates are essentially determined by the level of TCR engagement, with high Ag doses promoting strong accumulation of ERK-1 and -2. These results strongly suggest that signalling events originating from serially triggered TCRs are not simply sustained for the duration of time required for activation, but are gradually accumulated up to a threshold required for activation, as predicted by the temporal summation model.

From this model, it can be hypothesized that temporal summation occurs more efficiently when the TCRs (and the ligand-MHC complexes) are in close proximity at the cell surface. Increasing the local concentration of the receptors would facilitate their rapid engagement and consequently favour the summation of the signals up to the activation threshold. Therefore, the formation of the cSMAC, highly enriched in TCR molecules and signalling components, is believed to facilitate this process. Consistent with this idea, failure of partial agonists and antagonists to trigger full T cell activation appears to be related to their inability to induce the accumulation of TCRs at high density in the central cluster (348).

Another possibility is that low ligand density does not allow the formation of stable T cell/APC conjugates. The formation of the mature immunological synapse requires the stable interaction between a T cell and an APC. Experimental data suggest that a minimal density of ligand is necessary in order to form the mature synapse and that the density dependence is sensitive to the off-rate of the TCR-ligand interaction (348). Using real-time two-photon microscopy of intact lymph nodes, Bousso et al. showed that the binding of T cells to DCs bearing low densities of peptide is very inefficient as compared with binding to DCs bearing high densities of peptide (660). Therefore, it is possible that the failure to trigger T cell activation at low ligand density is due to an inability to form a stable synapse (and the high density accumulation of TCRs at the cSMAC), preventing the rapid and efficient serial engagement of TCRs.

5.2.1.2 Why is the TCR repertoire diversity restricted at low ligand concentrations?

Stimulation of T cells by SEA leads to the activation of T lymphocytes bearing different TCR Vβ family. Results presented in chapter 2 clearly demonstrate that these Vβs have different sensitivity for SEA, some of them being activated at very low doses of SEA while others need much higher concentrations (up to 4-5 log) to reach the same activation level. Although all these Vβs have relatively conserved structural features that allow their binding to SEA, they differ in critical residues involved in the interaction. These differences will most likely affect the binding to SEA and consequently the kinetic parameters of the interaction. As mentioned earlier, the temporal summation model predicts that a high affinity can compensate for a scarce display of TCR ligand. Therefore, based on the hierarchy of sensitivity to SEA established for all the responsive TCR Vβ families, we can predict the hierarchy of affinity of these Vβ for SEA (Figure 11). For example, Vβ22+ T cells can be activated, as measured by CD69 expression and proliferation, at very low doses of SEA (10^{-14} M, corresponding to less than ten SEA molecules/APC (661)), implying that Vβ22 binds strongly to SEA. This stable binding leads to the generation of individual signals having high amplitude and relatively long half-life that can compensate for the very limited numbers of individual signals generated at such low SEA concentrations. In contrast, Vβ1+ T cells require high doses of SEA for activation, probably because the low amplitude and the short half-life of individual signals must be counterbalanced by rapid, successive TCR engagement in order to allow for signal amplification.

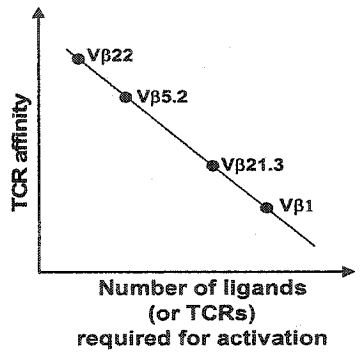


Figure 11. Inverse correlation between the binding affinity of the TCR for its ligand and the number of ligands (or TCRs) required for T cell activity

Although the data in chapter 2 have been generated using a superantigen for T cell stimulation, the same phenomenon of TCR repertoire narrowing at low antigen concentrations most likely occurs in classical antigen-restricted systems. The recognition of a pMHC complex by the TCR is characterized by degeneracy; a given TCR can recognize multiple different pMHC complexes and a given pMHC complex can be recognized by a panel of different TCRs (271). This degeneracy can be explained by the high structural flexibility of the TCR CDR3 loops that can undergo rearrangements to adapt to structurally different ligands (291). The multiple different TCRs specific for a given pMHC most probably have different affinities for the complex and, if so, T cells bearing these TCRs will also have different sensitivities for the ligand. Therefore, as described for SAgs, the level of presentation of an antigen (peptide) should also affect the size and the diversity of the responding T cell pool. Indeed, relationships between the number of pMHC complexes and the size, diversity, and avidity of the responding TCR repertoire have been established *in vitro* in conventional antigen-restricted systems (662-666). Low Ag presentation leads to the expansion of a smaller T cell population having a restricted TCR Vβ diversity but showing higher functional avidity as compared to T lymphocytes stimulated with high doses of peptide. These results suggest that low ligand concentrations limit the expansion of T lymphocytes by selecting the cells having the highest affinity for this ligand. Therefore, when a limited number of antigenic complexes are presented, high affinity T cells must be the only ones able to accumulate sufficient signals to attain the threshold for proliferation.

5.2.2 The influence of ligand density on T cell responses *in vivo*

The effect of antigen dose on the *in vivo* T cell response has been studied in several infection/immunization models (665;667-673). Overall, results and observations inferred from these studies correlate well with those obtained in *in vitro* experiments, but novel interesting observations were made.

Over a wide range of doses, the magnitude of the primary peptide-specific T cell response correlates with the density of antigen. This is consistent with *in vitro* data. However, because the clonal composition of the responding T cell population has never been compared between high and low Ag dose, it is not clear whether this reflects the preferential expansion of some higher affinity T cells at lower antigen densities or simply activation of a smaller number of cells due to a lower occupancy rate on the APCs. It is also possible that low Ag doses limit the duration of epitope presentation to T cells, leading to an abortive clonal expansion (674).

The size of the responding T cell population decreases at very high (supraoptimal) antigen doses. This unexpected result was observed with several antigens in different immunization models (670;671). It has been observed, at least *in vitro*, that supraoptimal Ag density can lead to the apoptosis of high avidity effector CD8 T cells by a mechanism involving TNF-α as well as the down-regulation of anti-apoptotic proteins such as bcl-2 and inhibitor of apoptosis (cIAPs)-1 (675-677). Similarly, T cells stimulated with high doses of SAgs show abortive stimulation and undergo apoptosis after few rounds of divisions, both *in vitro* (661;678) and *in vivo* (679). Moreover, infection of mice with high doses of virus leads to decreases in CTL function, exhaustion of the clonally-expanded T cells and viral persistence (680-685). It is hypothesized that when large doses of virus are challenged *in vivo*, viral Ags might be presented at an increased level on the cell surface of APCs, possibly causing the deletion of high avidity CTLs and inadequate control of viral spread by low avidity CTLs (686).

What could cause the death of T cells stimulated with supraoptimal doses of ligand? It is well described that strong TCR ligation is accompanied by massive TCR down-modulation, a mechanism believed to ensure termination of the TCR signals, thus avoiding T cell hyperactivation and possibly unchecked immune responses (333;687;688). Results from our laboratory (see figure 5 in chapter 2 and (661)) have shown that T cell stimulation with high doses of SEA leads to rapid extensive TCR internalization that is more prominent in higher avidity T cells (Vβ22+ T cells in the case of SEA). At very high SEA doses, cell-surface TCR expression becomes undetectable for 48 to 72 hours as measured by anti-TCR staining. Supraoptimal SEA concentrations also induce an inhibition of cytokine (IFN-γ and IL-2) secretion by the activated T lymphocytes. A similar relation between extensive TCR internalization and inhibition of IL-2 secretion has been made in a peptide model (278). Interestingly, it has been shown that disruption of TCR stimulation by removal of peptide antigen or by using a blocking antibody results in the immediate loss of cytokine production, consistent with the hypothesis that continual TCR stimulation is needed to maintain cytokine synthesis (355;475;689). It is possible that the massive TCR internalization occurring following high dose SEA stimulation causes the complete depletion of cell-surface TCRs on activated T cells and prevents the continuous engagement of TCRs necessary to maintain cytokine synthesis. Supporting this idea, T cells from Cbl-b-deficient mice, which have impaired TCR down-modulation, show increased production of IL-2 (238;690). It can be hypothesized that the synthesis of genes involved in protection from apoptosis (such as bcl-2, bcl-xL, cIAPs) are also dependent on sustained TCR-mediated signals and that premature termination of signalling due to a rapid TCR down-regulation prevents the continuous expression of these genes and therefore provokes the apoptosis of the activated cells. Indeed, it has been shown that prolonged (but not brief) T cell stimulation leads to increased expression of bcl-xL (691).

The density of antigen influences the overall affinity/avidity of the memory and recall (but not the primary) T cell population. Surprisingly, it appears that the functional and structural avidities of T cells recruited into a primary response are identical, regardless of the epitope density used for immunization (665;669). However,

an inverse relation between peptide dose and the apparent receptor affinity of the T cells (as measured by tetramer binding) is observed in the memory population and after a secondary stimulation either *in vivo* or *in vitro*. The relatively short duration of epitope presentation by peptide-pulsed APCs (692-694) and the recent demonstration that the contraction phase of the primary response is programmed and antigen-independent (695) argue against a possible epitope density-based selection of T cells during the contraction phase.

A recent report showing an increase in TCR avidity following T cell activation could provide an explanation to these seemingly incompatible observations (478). It is possible that intrinsic differences in TCR affinity during the primary response are masked by the overall increase in TCR avidity due to cholesterol-dependent membrane reorganization of the TCR in activated T cells. This compensatory mechanism would minimize the differences in affinity of the activated T cells. If this TCR reorganization is not permanent, differences in TCR affinity could become apparent in resting memory cells. However, the fact that differences in TCR affinity can also be observed during recall response implies that this compensatory mechanism becomes inoperative following secondary challenge.

Another possibility is that high-affinity T cells are more susceptible to CTLA-4-mediated inhibition during the primary response (696-698). This model is strongly supported by recent experimental data demonstrating that the accumulation of CTLA-4 at the immunological synapse is finely regulated by TCR signal strength (361). This would restrict the expansion of high affinity T cells and allow for greater representation of cells bearing lower affinity TCRs, and consequently a greater diversity in the early stages of the T cell response. By decreasing, but not eliminating, the competitive advantage of dominant clones, CTLA-4 would help to maintain the breadth of diversity in the antigen-specific effector population. Again, the fact that differences in TCR affinity can also be observed during recall response indicates that this inhibitory mechanism does not operate following secondary challenge.

Recent *in vivo* evidence suggests that persisting memory T cell populations have a greater affinity for the specific peptide/MHC ligand compared to the naïve population, a phenomenon termed T cell affinity maturation (665;699-702). In other systems, the TCR repertoire of antigen-specific T cells appears to remain stable throughout the different phases of the immune response (primary, memory and recall), suggesting that affinity maturation occurs in specific circumstances (449;703-705). How and why could high affinity T cells be preferentially selected to become memory over the pool of responding cells?

A possible explanation may be that high affinity T cell clones are activated at much lower concentrations of antigen, allowing them to proliferate earlier during the primary response and to rapidly become prevalent over low affinity T cells. Such a kinetic advantage could favour, in a purely statistical manner, their selection to become the predominant memory cells in a model of stochastic selection from a pool of specific T cells. Indeed, it has been shown that the contribution of a T cell clone to the immune response is mostly determined by the time of first cell division following antigen encounter (706).

It has been demonstrated that T cells compete for access to antigen presenting cells; competition for a limited number of antigen/MHC complexes is believed to influence the repertoire selection by favouring high affinity T cells (707-709). This selection process would be particularly relevant when the number of specific antigen/MHC at the surface of APCs is low, as suggested by *in vivo* results showing that the average TCR affinity of the memory population is an inverse function of antigen dose during the primary response (665). Moreover, competition for a limited number of surface interactions may also underlie the establishment of immunodominance during the immune response (710). A similar phenomenon of clonal competition has been observed during the B cell responses (711).

The extent of T cell competition for access to APCs most likely depends on T cell precursor frequency. It has been suggested that affinity maturation is not observed during primary responses because the frequency of any given clone is too small to have any

significant effect on other responding T cells (712;713), explaining why competition and affinity maturation are generally observed following multiple infections/immunizations (665;699-702;714;715). In agreement with this hypothesis, it has been reported that the competitive ability of T cells increases in proportion to their frequency (709;716). A corollary of this hypothesis is that increasing the number of antigen-loaded APCs should minimize the effects of T cell competition (707).

Using different experimental model systems, several groups have demonstrated that T cells remove and internalize MHC molecules and other proteins from the surface of interacting APCs (717-724). Interestingly, Huang *et al.* made a strong correlation between the affinity of the TCR for its cognate pMHC and the ability of the T lymphocytes to remove antigen from the APC. This finding agrees well with the observations that high affinity T cells are better competitors than low affinity T cells and suggest that this mechanism may represent the driving force behind T cell affinity maturation (713). Higher affinity T cells down-modulate antigenic complexes from the APC, making it more difficult for lower affinity cells to detect antigen, accounting for the exclusion of the lower affinity T cells from the activated pool. As mentioned earlier, this phenomenon of exclusion must be more prominent in situation of limited number of ligands at the surface of the APCs.

High affinity T cell clones may alternatively receive a qualitatively different signal compared to low affinity clones: the fate of T cells would then be decided upon first encounter with the antigen. The temporal summation model discussed earlier predicts that the kinetics of peptide/MHC-TCR interactions determines whether the threshold for activation of different intracellular signalling pathways is reached and thereby direct the scope of the effector response mobilized. Differences in the quality of the signal received by the low versus high affinity T cells may therefore be hypothesized to drive the development of memory cells or simply influence T cell susceptibility to AICD. Because the selective advantage of higher affinity T cells would only be apparent after memory formation, this could explain why affinity maturation is not apparent during the primary response.

5.2.3 TCR affinity and T cell fate

The study of APLs has generated an increased interest in the functional consequence of TCR engagement by its ligand and has provided valuable information about the factors ruling TCR ligand discrimination and T cell activation. More specifically, APLs allowed making correlations between the affinity of the TCR/pMHC interaction and the extent of T cell activation. In general, a good correlation has been demonstrated between the kinetics of binding, in particular the rate of dissociation of the TCR from the MHC/ligand complex, and the strength of T cell activation. Strong agonist ligands have the longest half-life of the complexes, partial agonists and antagonists have an intermediate half-life and null ligands dissociate very rapidly from the TCR (725). It is now well accepted that T cell fate, from development to death, is predominantly governed by the affinity of the TCR-ligand interaction.

5.2.3.1 TCR affinity and thymocyte selection

Whether a developing thymocyte becomes positively or negatively selected is thought to be determined by the avidity/affinity of its TCR for pMHC complexes expressed in the thymus (167). Affinity measurements support the idea that TCR affinity for positive selection ligands is lower than for negative selection ligands (275;280;726). Moreover, using real-time microscopy, Richie *et al.* demonstrated that negative selection (but not positive selection) is associated with efficient conjugate formation and rapid recruitment of $p56^{Lck}$ and CD3 ζ to an immunological synapse (727). Because of their short occupancy time, low-affinity ligands would be limited to inducing only early TCR signals and thus lead to positive selection. High-affinity ligands, on the other hand, contact the TCR for a longer time and induce late TCR signals that initiate negative selection. It is presumed that differences in the affinity of TCR-ligand interaction are translated into differences in signal strength which, in turn, regulate the outcome of thymocyte selection.

Using a defined set of peptide variants of the LCMV-gp epitope, Mariathasan *et al.* made a correlation between the degree of TCR internalization, the level of calcium flux and the outcome of the selection process (728). Strong agonist peptides that mediate strong TCR down-modulation also trigger elevated and sustained calcium flux and are effective at mediating negative selection. In contrast, partial agonist/antagonist peptides that induce suboptimal TCR internalization trigger a transient calcium flux and promote positive selection. A similar relationship between the calcium flux and the outcome of thymocyte selection has been established by three other groups (729-731).

Studies using calcineurin inhibitors such as FK506 and cyclosporin A have shown that Ca^{2+}/calcineurin pathways are involved in the positive and negative selection processes (732-735). A recent report suggests that a protein called Ca^{2+}/calmodulin-dependent protein kinase type IV/Gr (CaMKIV/Gr) could be responsible for Ca^{2+} signal discrimination (736). In the absence of this protein, ligands that would normally induce negative selection were positively selected and large numbers of low-affinity peptides that normally induce positive selection are rendered functionally null, leading to impaired positive selection. The authors showed a defective induction of Ca^{2+}-regulated genes in CaMKIV/Gr-deficient thymocytes; for example, the expression of CD40 ligand, TNF-α and the orphan steroid receptor Nur77 (all of which have been implicated in negative selection) was abrogated (737-739).

Another model getting increased support is a differential MAP kinase activation model. This model predicts that positive selection results from a low, sustained level of ERK activation, whereas negative selection occurs in response to a large burst of ERK activation concomitant with JNK and p38 activation (167). Supporting this model, analysis of the kinetics of ERK activation showed that peptides driving negative selection generate a short burst of ERK activity while positive selection peptides generate a lower but more long-lived pattern of ERK phosphorylation (740-742). The activation of the ERK cascade promotes contrary cellular responses (including cell proliferation, cycle arrest, differentiation, senescence, or apoptosis) depending on the kinetics of ERK activity (740;743). It is possible that a low-affinity interaction that is associated with a

sustained activation of ERK results in proliferation and further differentiation of thymocytes (positive selection) whereas a high-affinity interaction, associated with a transient activation of ERK, results in apoptosis and negative selection. Therefore, different patterns of ERK signalling could activate different transcription factors and effector molecules, some promoting survival and differentiation while others inducing apoptosis.

5.2.3.2 TCR affinity and the regulation of naïve T_H differentiation

It has been demonstrated in several independent *in vitro* and *in vivo* model systems that providing a low potency signal to the TCR favours T_H2 differentiation while a strong TCR signal shifts the balance toward T_H1 polarization (744-746). Priming CD4 T cells with optimal doses of an agonist peptide induces the development of T_H1 cells; in contrast, priming with altered peptide ligands which have low affinity for the TCR preferentially induces T_H2 polarization (747-749).

Insight into the molecular basis by which high and low affinity ligands generate different signals that are interpreted by the T cell to initiate the transcriptional program for T_H1 and T_H2 differentiation have been provided by studies on the regulation of members of the NFAT family. The activity of these transcription factors is finely regulated by the level of calcium mobilization within a cell (750;751). Calcium increases following TCR stimulation leads to nuclear translocation of these factors (by phosphorylation) whereas a drop in calcium levels results in their exportation from the nucleus to the cytoplasm (by dephosphorylation) (752;753). Consequently, the extent of calcium mobilization initiated following TCR ligation may affect NFAT translocation and, in turn, NFAT-dependent gene transcription.

Experimental data from independent groups revealed that T cells from NFATc-deficient mice have impaired IL-4 production and show decreased T_H2 differentiation (754-756) while mice deficient in NFATp/NFAT4 demonstrate a profound increase in IL-4 production and allergic inflammation (which is strongly associated with T_H2) (757-759).

The increase in IL-4 production in these mice is attributed to unopposed activity of NFATc which was highly activated in T cells from these mice (758). Recently, Bottomly and coworkers showed that the potency of TCR signalling differentially affects the balance of NFATc and NFATp DNA-binding activity, thereby regulating IL-4 transcription (760). Stimulation of T cells with a low affinity peptide results in high levels of nuclear NFATc and low levels of nuclear NFATp whereas both are found at high levels following stimulation with a high affinity peptide (760). Because the high affinity peptide promotes a stronger and more sustained calcium mobilization as compared to the low affinity peptide, these results indicate that NFATp is less sensitive to calcium/calcineurin activation then NFATc. This is a good example of how signal strength, as determined by TCR affinity, controls the level of a second messenger (calcium), which in turn controls the activation of specific transcription factors (NFATc and NFATp), which ultimately dictates T cell differentiation and fate (T_H polarization).

5.2.3.3 TCR affinity and T cell effector functions

As illustrated in figure 10, T cell activation is not simply an on/off process; different levels of TCR triggering are necessary to reach the activation threshold for different effector functions. According to the temporal summation model of T cell activation, the affinity of the TCR-ligand interaction will influence the extent of signal accumulation (mostly by affecting the quality of the individual TCR-mediated signals), which, in turn, will dictate the array of effector functions displayed by the T lymphocytes. Similarly, it has been demonstrated that the BCR triggers a variety of biological responses that differ depending upon the affinity of the antigen (325;761;762).

Using different experimental model systems, several independent groups have shown that T cells activate effector functions according to a ligand avidity-related hierarchy (666;676;763-770). Even though clone-specific differences are sometimes observed, a hierarchy in effector function can be established (figure 12). While cytolysis is the easiest function to trigger in T lymphocytes, sustained cytokine (especially IFN-γ and IL-2) production and extensive cellular proliferation usually requires high antigen

concentration and/or a high affinity ligand, co-stimulation and long-term maintenance of the immunological synapse. Consistent with the idea that strong TCR engagement is necessary for proliferation and especially cytokine secretion, it has been shown that these functions are triggered when 20 to 50% of TCRs are internalized (766;767). Based on the temporal summation model, two non-mutually exclusive mechanisms can explain why different effector functions are elicited at different TCR avidity. First, it is possible that the functions do not require the same accumulation of second messengers to be triggered, some of them being initiated by a low and transient increase in second messengers while others need a strong and sustained accumulation to be elicited (as illustrated in figure 10). Alternatively, it is possible that different second messengers (or combination of) control the triggering of each effector functions. If these signalling intermediates need different levels of TCR engagement to accumulate, effector functions depending on the accumulation of these intermediates will also be triggered at different levels of TCR engagement.

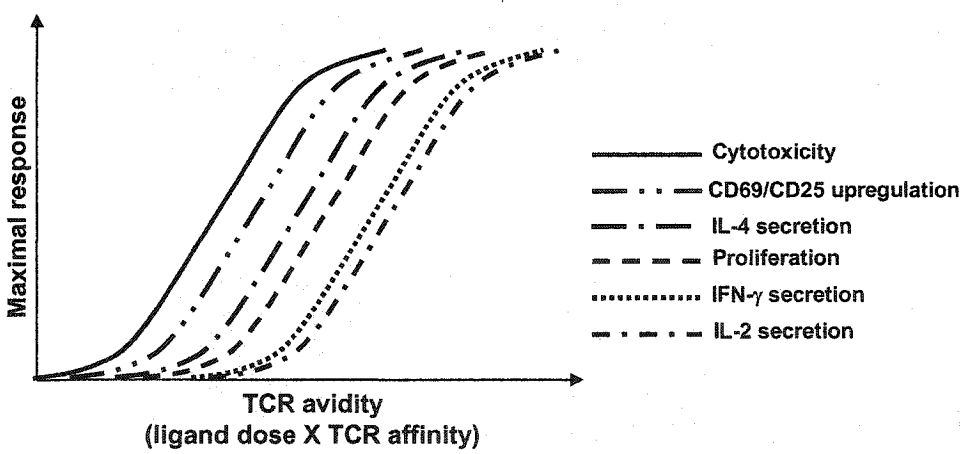


Figure 12. T cell effector functions have distinct thresholds.

We derived similar conclusions from our data with SEA. An "optimal" SEA concentration leads to the simultaneous CD69/CD25 upregulation and proliferation of all responsive TCR Vβ cells. However, we observed drastic differences between these TCR Vβ in their ability to secrete IL-2 at the "optimal" dose. Comparison of the SEA-induced

TCR internalization revealed that the capacity to produce IL-2 is related to the level of TCR down-modulation; Vβ22+ T cells, which show strong TCR internalization, constitute good IL-2 producers but almost no IL-2 was detected in Vβ1+ and Vβ21.3+ T cells, which showed very limited TCR internalization. Indeed, several reports have demonstrated that the extent of TCR down-regulation correlates with the affinity of the TCR-ligand interaction (278;279;771), the strength of TCR-mediated signals (688;772) and the induction of a variety of effector functions (767;773).

Using MHC class I tetramers, Hafler and coworkers isolated several CTL clones (having different TCRs) reactive to a single viral epitope from HTLV-1 (774). Functional studies have revealed that these clones display similar cytotoxic activity but different degrees of proliferative response and cytokine secretion. Because all the clones must have been exposed to similar stimulating conditions during the primary immune response, the heterogeneity in terms of proliferation and cytokine secretion most likely reflects intrinsic differences in TCR affinity. Therefore, it appears that a polyclonal antigen-specific T cell population comprises clones having different functional behaviours, most likely due to their different TCR structure that confers them different affinities for the antigen.

There are some examples highlighting the importance of the TCR affinity in the outcome of the immune response. Using a model of *Leishmania major* infection, Malherbe *et al.* showed that resistance to infection is associated with the expansion of high affinity CD4 T cells whereas susceptibility is associated with a low affinity TCR repertoire (775). High affinity CD4 T cells from a resistant mouse strain (B10.D2) produced higher levels of IFN-γ (T_H1) and lower levels of IL-4 (T_H2) as compared to low affinity CD4 T cells from MHC-matched susceptible mice (BALB/c). It is well known that resistance and susceptibility to *Leishmania* correlate with the development of T_H1-and T_H2-dominated responses, respectively (776). As mentioned earlier, many studies have suggested that the strength of signal through the TCR may influence the generation of T_H1 and T_H2 effector lymphocytes, both *in vivo* (777) and *in vitro* (747;749).

A major limitation to the efficacy of cancer vaccines is that endogenous T cell responses against tumour antigens tend to be relatively weak. In some cases, the weak T cell responses to tumour antigens appear to be related to the low affinity of the pMHC for the TCR. It is believed that high-affinity tumour-specific T cells are actively tolerized via anergy or deletion, thereby leaving a functional repertoire consisting of T cells bearing TCRs with low affinity for MHC-tumour peptide complexes. To test this hypothesis, Pardoll and colleagues generated tumour peptides with mutations at TCR binding residues, leading to a more stable TCR-pMHC complex (635). Immunization experiments with the mutated epitope led to the activation and expansion of polyclonal T cell responses specific for the wild-type antigen and to better antitumour immunity. The mechanism by which the modified peptide enhances the destruction of tumour bearing the wild-type remains unclear. Nevertheless, one possibility is that naïve tumour-antigen specific T cells might not be sensitive enough to detect the native/endogenous tumour antigen, but that primary recruitment of these clones using the modified high-affinity epitope leads to an increase in the clones' functional avidity, henceforth allowing them to detect the low affinity wild-type antigen (478;479). Consequently, modification of low immunogenic peptides at TCR contact residues with the aim to increase their affinity for the TCR could be an advantageous strategy to activate a potent and functional T cell repertoire in vaccination protocols.

It has been demonstrated in different models of immunization/infection that high avidity cells are much more effective than low avidity cells in reducing viral load and in destroying tumour cells (662)(663;778-782). High affinity CD8 T cells appear to exploit three complementary mechanisms to provide a better protection (778;779). First, because they can recognize lower density of antigen, they attack infected cells earlier in the course of viral infection. Second, they lyse infected cells more rapidly than low affinity cells at any antigen dose. Also, whether early or late during the course of infection, it may be hypothesized that high-affinity T cell clones are capable of targeting infected cells that naturally present fewer antigenic complexes, thus having the potential of sterilizing what would otherwise have been a cell type exploited for escape. These three mechanisms probably contribute to preventing the spread of the pathogens within the host.

5.2.4 The TCR repertoire: balancing efficiency and diversity

The response to a given antigen is normally composed of multiple T cell clones, each with distinct TCR-pMHC kinetics. Therefore, a spectrum of TCR affinities most probably exists in the pool of responding cells. As previously discussed, T lymphocytes having the highest affinity for an antigen appear to possess a competitive advantage over T cells of lower affinity for the same antigen, as manifested by the formers' more rapid and efficient ability to be clonally expanded. This kinetic advantage leads, in certain circumstances, to the phenomenon known as T cell affinity maturation. Moreover, as discussed above, there is accumulating evidence that high affinity T cells are more efficient at clearing infections as compared to low affinity T cells. These data support a model in which the immune system should favour the exclusive expansion of high affinity T lymphocytes in order to efficiently fight infections. However, T cell affinity maturation does not seem to take place in every type of infection and is generally only observed during memory and recall responses. Thus, it appears as though the immune system has developed mechanisms to avoid the uncontrolled domination of specific T cell clones over the others in order to maintain a certain level of diversity. These mechanisms hinge on several parameters, including:

1. **Optimal conditions of T cell priming.** Dendritic cells are specialized in the presentation of antigens to T cells. As previously reviewed in chapter 1, DCs have developed several mechanisms in order to efficiently uptake, process and present antigenic peptides to T cells. They also express high levels of co-stimulatory molecules. Moreover, in the secondary lymphoid organ environment, T cells can simultaneously interact with multiple antigen-loaded DCs. All these mechanisms, by optimizing the conditions of T cell stimulation, contribute to T cell diversity by favouring the recruitment of both high and low affinity T cells in the immune response.

2. **Specific inhibition of high affinity T cells.** As discussed above, it appears that high affinity T cells are more susceptible to CTLA-4-mediated inhibition of T cell activation. CTLA-4 engagement and signalling restrict T cell expansion by reducing the synthesis of proteins involved in cell cycle progression such as cyclin D3, cyclin-dependent kinase (cdk) 4 and cdk6 (783;784). Therefore, the competitive advantage of high affinity T cells seems to be counterbalanced by the CTLA-4-mediated inhibition of their clonal expansion. This phenomenon was highlighted in a recent study by Gray *et al.* (778). They demonstrated that high affinity T cells dominate the response at early time points following infection but that their dominance is extinguished at later time points due to the preferential expansion of lower affinity T cells.

Although the attenuation of affinity maturation in order to maintain TCR diversity in the response may seem counterintuitive, it could serve important functions. First, low affinity T cells may exhibit greater cross-reactivity to modified antigenic peptides. Analyses of TCR sequence and structure have revealed that high affinity TCRs are often characterized by more rigid CDR3 loops that possibly limit their cross-reactivity with other epitopes (291;785). Maintaining a diverse T cell repertoire could improve the ability of the immune system to respond to mutated epitopes generated by an infectious agent trying to escape the immune response. Mounting an oligoclonal or a monoclonal T cell response could give an obvious advantage to the pathogen, since mutation of the dominant epitope would allow it to avoid T cell recognition by decreasing the TCR-pMHC affinity (786). Indeed, it has been shown that the ability of SIV-infected monkeys to recognize epitope variants is related to the development of a diverse, polyclonal TCR repertoire against the wild-type epitope (787). Moreover, resolution of some viral infections is strongly associated with the development of a very large CTL response targeted against multiple viral epitopes (788;789). Second, it is now well described that the TCR affinity may dictate the array of effector functions displayed by the T cells. More specifically and as previously discussed, the cytokine expression profile of responding T cells may be determined by the affinity of their TCRs for pMHC complexes and may influence the outcome of an infection (375;775). Therefore, the expansion of

low affinity T cells could be needed to produce specific cytokines or other mediators required to eliminate certain types of infection, or again to mobilize an array of multiple effector functions in order to counter a pathogen's dissemination.

5.3 Heterogeneity in the CD4 T cell response: from primary T cell expansion to memory T cell development and differentiation

Results presented in chapter 2 clearly demonstrate the existence of clone-specific functional heterogeneity in CD4 T cells undergoing T cell expansion. This heterogeneity, which reflects differences in gene expression profiles, is most probably due to qualitative and quantitative differences in the signals received by the different T cell clones. How can such heterogeneity in gene expression influence the development and the differentiation of T cells into memory? A link between the expression of specific genes by clonally-expanded T cells and their ability to become memory has been established in transgenic mouse models.

Jacobs and Baltimore have developed an original transgenic mouse system that allows the permanent labelling of activated and memory T cells (790). In these mice, the Cre-recombinase gene was introduced under the control of a truncated human granzyme B promoter. A placental alkaline phosphatase (PLAP) reporter gene was also introduced such that its synthesis is induced only in T cells and only following a Cre-mediated recombination event. Activation of the T cell normally leads to induction of the granzyme B gene promoter; thus, in these double-transgenic mice, it ultimately results in the transduction of human PLAP that permanently labels the T cell having undergone activation. Surprisingly, both during the peak of an LCMV infection and >30 days after the infection, only a small persisting fraction (5%) of activated T cells are $PLAP^+$ and thus show the potential to become long-term memory. Though these "pre-memory" T cells display functional effector properties similar to those associated with effector T cells, this subset of T lymphocytes having probably received a distinct signal early during the immune response will persist to memory.

In a different experimental system, the expression of IL-2 by a subpopulation of activated naïve T cells has been linked to an enhanced memory/effector function (791). Using an IL-2 promoter/GFP-reporter transgenic model, it was shown that $CD4^+$ T cells reaching the activation threshold associated with IL-2 gene transcription during the primary response later have an enhanced ability to produce effector cytokines, provide better B cell help and show increased sensitivity to antigenic stimulation as compared to those that did not reach the threshold. Using the same system, they have recently demonstrated that long-term, stable interactions between a T cell and a DC is required for IL-2 expression by the T cell (792). Transient interactions with DCs lead to T cell activation and IL-2 responsiveness, as measured by CD25 upregulation, but fail to induce IL-2 gene expression.

5.3.1 General models for memory T cell generation

The mechanisms underlying memory T cell differentiation are still poorly understood. Two theoretical frameworks attempting to explain the generation of memory have been generated from experimental results. The first is a linear differentiation model whereby cells undergo necessary maturation through an effector stage before becoming memory; the second is a dichotomic differentiation model whereby memory cells are made such without having necessarily been effectors at any point. These are illustrated in figure 13.

Evidence supporting the dichotomic model is mostly provided by the two transgenic systems described above (790;791). Support for the linear differentiation model comes from independent groups that have demonstrated that, upon encounter with antigen, T cells must undergo rounds of division before exerting effector function and having the potential of becoming memory (483;483;793;794;794;795;795). These experiments typically involved the sorting of CFSE-labeled cells having undergone set numbers of *in vitro* peptide-driven cell divisions and their tracking following re-infusion into syngeneic hosts. Adoptive transfer experiments using cells from TCR- transgenic mice also support this model (445;796;797). For all these studies, it is unclear whether all transferred cells truly were constituted of a homogenous pool of identical effector cells or whether the

seeding of memory was the result of a subset of the transferred cells sharing phenotypic properties with transferred effectors.

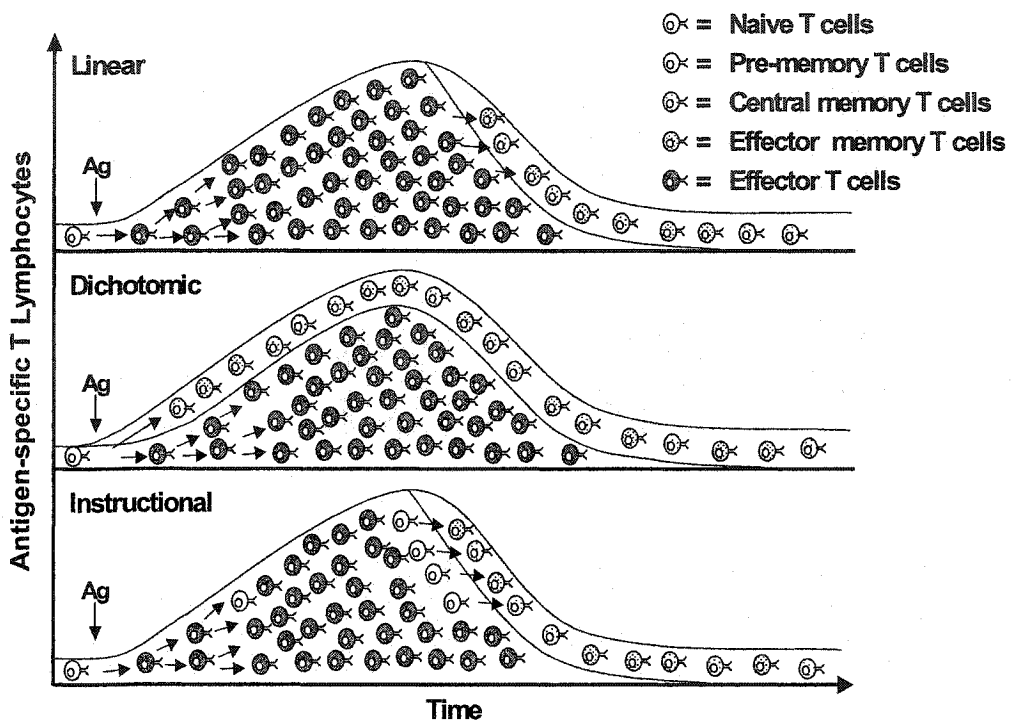


Figure 13. Models for memory T cell generation. **A.** *Linear differentiation model. Following antigen encounter, naïve T cells proliferate and differentiate into effectors; after antigen clearance, only a small proportion survive and constitute the memory T cell pool.* **B.** *Dichotomic model. A subset of naïve T cells directly differentiates into memory without acquiring effector functions. At the end of the immune response, effector cells undergo apoptosis while the memory subset persists.* **C.** *Instructional model. A subset of naïve T cells receives a signal that drives them to differentiate into "pre-memory", further differentiation requires proliferation. These "pre-memory" cells form the pool of T_{CM} and T_{EM} memory T cells following antigen clearance.*

5.3.1.1 A matter of perspective...

To date, the key difference between experiments supporting a linear differentiation model and those supporting a dichotomic model is in the original property used to isolate the cells to be characterized for their ability to seed memory. Experiments using cells sorted on the basis of bromodeoxyuridine (BrdU) or CFSE-labelling rely on their proliferative history but fail to demonstrate the functional homogeneity of the sorted population. While enrichment of measured effector function in the sorted populations correlates with enrichment of memory precursor content, direct demonstration that the cells exerting the function are the same which later seed memory remains to be performed. Conversely, experiments relying on the study of T cell populations selected on the basis of their demonstrated function are limited by the transgenic nature of the systems used and the unknown history of individual cells. Finally, our limited knowledge of the complexity of factors that modulate the promoters and enhancers that direct the expression of tracer-proteins in transgenic reporter systems complicates interpretation of studies making their use. Altogether, the complexity of the memory phenotype with regards to function as defined either by proliferation or by which cytokine/effector molecule is upregulated, is such that even the most elegant systems available force the bias of which property to primarily target. Critically, the integration of key elements from the two primary models explaining the formation of T cell memory as described above allows for a more refined model, the instructional differentiation model (Figure 13c).

5.3.1.2 The instructional differentiation model

Recent studies have shown that a brief initial antigen encounter is sufficient to induce naïve CD8 T cells to expand and mature to differentiated effectors that display memory properties *in vivo* (798;799). Though a few hours of antigen stimulation in the presence of APCs expressing B7 appear to be sufficient, other factors contributing to the appropriate co-stimulation remain to be identified (674). The results obtained by various

groups investigating the CD4 T cell response suggest that CD4 T cell proliferation may also be induced by a brief encounter with antigen, but that differentiation requires protracted exposure (800;801). Both for CD4 and CD8 T lymphocytes, signals of a certain nature or quality may therefore confer some cells with the potential to become memory, a potential that is then exploited only if these "pre-memory" cells receive further signal. Supporting this idea, it was demonstrated that T cell "fitness", as defined by enhanced effector functions, survival and homeostatic cytokine responsiveness, is determined by the strength of the signal initially received by the cell (674). The maturation of memory cells would thus be a multi-step process requiring a timely succession of key stimuli.

Critically, studies by Manjunath et al. have demonstrated that the generation of memory T cells does not require prior linear differentiation through an effector stage: alone, the cytokine environment at the time of recruitment may be sufficient to bias maturation of the T cells as central or effector memory (802). These experiments made use of an original transgenic system of T-GFP mice, in which a green-fluorescent protein (GFP) marker was introduced under the control of the CD4 promoter and proximal enhancer (803). Original observations using these mice were that GFP was expressed in naïve CD4 and CD8 T cells but lost from a significant proportion of CD8 T cells having been stimulated, and that the effector properties of cytotoxicity and IFN-γ secretion were restricted to GFP^- cells. While *in vitro* restimulation of the GFP^- cells in the absence of exogeneous cytokines resulted in their apoptosis, exogenous provision of IL-2 rescued these cells and supported their proliferation (803). In a follow-up study, T-GFP mice were crossed to TCR transgenic mice in order to study T cell differentiation in an antigen-dependent manner. *In vitro* peptide-specific stimulation of the naïve double transgenic CD8 T cells in the presence of high concentrations of IL-2 resulted in the formation of GFP^- effectors that did not express CCR7. Subsequent resting of the cells (7 days) and switch to IL-15-containing medium resulted in their apparent reversion to a memory phenotype that nevertheless did not express GFP or CCR7. Meanwhile, *in vitro* stimulation of naïve CD8 T cells in the presence of low doses of IL-2, or in the presence of IL-15 directly resulted in the formation of memory cells that secreted IFN-γ, lacked

immediate cytotoxic function and phenotypically remained positive for GFP and CCR7 expression. Thus, distinct pools of memory CD8 T cells can be generated by linear differentiation through an effector stage, or directly as per the dichotomic differentiation model.

Conceptually, it may therefore be possible to reconcile data supporting the linear and dichotomic models discussed above with an instructional differentiation model for the generation of memory T cells (figure 13c). This model is based on the premise that the stimuli received by different naïve T cells are variable both in nature and intensity. This variability would result from differences in signals provided by different affinity/avidity of the interaction between peptide/MHC and TCR, by co-stimulation and the cytokine environment in which the cells are engaged. While summation of the memory-inducing signals through multiple descendent generations of a precursor cell would result in a "linear" pattern of memory generation, provision of the necessary signals to a non-proliferating T cell would result in a pattern compatible with an intrinsic dichotomy of memory T cells (figure 13). A recent report by Rafi Ahmed's group strongly supports this model (804). They directly showed that memory cell precursors exist during the effector phase (by day 8 post-infection) but they do not display functional properties ascribed to memory T cells, such as the ability to proliferate to homeostatic signals and to divide rapidly and extensively following antigen reencounter. These properties appear to be gradually acquired over the weeks following viral clearance.

The existence of distinct CD4 effector subpopulations with differential capacity for generating long-term memory T cells has been highlighted in a recent report (805). By assessing the secretion of IFN-γ, Wu *et al.* discovered that T_H1 memory and effector function are provided by two different subpopulations of T_H1 cells that develop during a primary immune response: IFN-γ^- cells with long-term memory function and short-lived IFN-γ^+ cells with immediate effector function. The secretion of IFN-γ appears to abrogate the proliferation and to sensitize CD4 T cells to apoptosis, a concept strongly supported by a wealth of experimental data (442;443;806-808).

5.3.2 Effector and memory T cell differentiation during the immune response

The data reported in chapter 3 highlighted the heterogeneity, in terms of proliferative capacity and effector functions, of the memory CD4 T cells subsets defined by CD45RA and CCR7 expression. Moreover, we clearly demonstrated that T cells belonging to a given subset (most notably the CD45RA-CCR7- effector memory CD4 T cells) also show heterogeneity in their ability to secrete IL-2 and IFN-γ. A refinement of the instructional model described above can be proposed (figure 14) based on the results presented in chapter 3 and on the following observations reported in the literature (for CD4 and/or CD8 T cells, in mouse and human models):

- T_{CM} have increased proliferative potential and have a greater ability to persist *in vivo* as compared to T_{EM} (809);
- Memory T cell precursors exist in the effector population (790;804);
- CD4 T cells migrating to peripheral tissues are more differentiated (i.e. many cell divisions, high IFN-γ production and low IL-2 production) as compared to those in the secondary lymphoid organs (810).
- IFN-γ-producing effector T cells are more prone to apoptosis and are impaired in the ability to become long-term memory T cells (805);
- Memory T cell development is linked to the presence of cells with the capacity to produce IL-2 (791;811);
- The ratio of IL-2- to IFN-γ-producing cells increases with transition into the memory phase of the immune response (811);
- High viral loads and chronic infection lead to the generation of T cells with low proliferative capacity and defective IL-2 production (682;683;811;812).

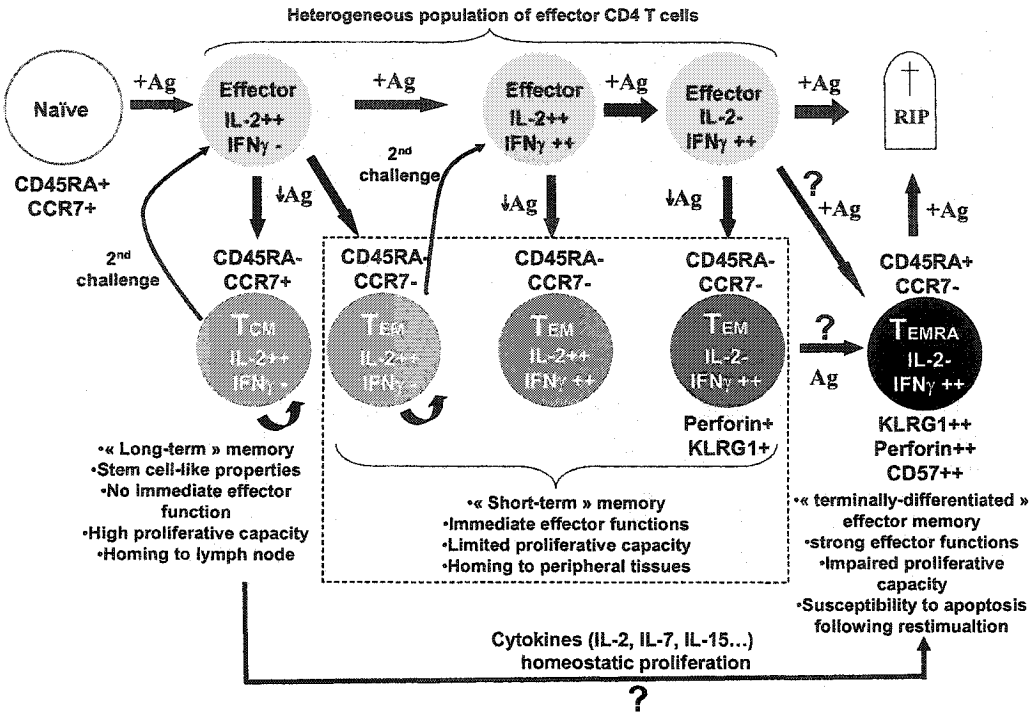


Figure 14. Model of memory CD4 T lymphocytes development and differentiation. *Antigen-mediated stimulation of naïve T cells leads to their expansion and to their differentiation into effector T cells. The duration and the level of antigenic stimulation to which the T cells are exposed primarily determine the differentiation of the effector cells into IL-2- and/or IFN-γ-producing cells and their further development into central or effector memory cells. While a strong stimulation appears essential to promote T cell fitness, prolonged or chronic exposure to a high antigen load drives effector T cells to terminal stages of differentiation and ultimately to cell death. The progression of cells through the stages of differentiation is accompanied by a gain in some effector functions such as expression of IFN-γ and perforin, and by a progressive loss of proliferative capacity and IL-2 secretion.*

As previously mentioned, this model is based on experimental data obtained in CD4 and CD8 T lymphocytes. Although much is known about the dynamics of CD8 T cell responses, the magnitude, specificity and duration of $CD4^+$ T-cell responses have only recently been directly examined. Also, it is important to mention that significant differences in the homeostasis of these two T cell populations have been observed. These include:

- Naïve CD4 T cells need longer time of antigen exposure and are more dependent on co-stimulation to get fully activated (656;799;813).

- The magnitude (burst size) of the $CD8^+$ response vastly exceeds (~ 20-fold) that of the $CD4^+$ response (413;814). In fact, CD8 T cells divide to a greater extent and faster (~15 divisions, 6 h per division) than CD4 T cells (~9 divisions, 9–20 h per division).
- The contraction phase is delayed in CD4 T cells. Typically, about 90–95% of the antigen-specific $CD8^+$ T cells die from apoptosis during a 1–2-week period whereas 50 days are required to remove a similar percentage of $CD4^+$ T cells (413;815).
- In mice, CD8 memory T cell numbers remain stable for prolonged periods of time (for up to 900 days) whereas the half-life of CD4 memory T cells has been evaluated to be approximately 400 days (413).
- The distribution of CD4 and CD8 T cells among the memory and effector memory subsets largely differs (482). For example, a large population (20-30% of CD8 T cells) of "terminally" differentiated (CD45RA+CCR7-) CD8+ T cells is found in healthy blood donors whereas this population is rarely detectable in CD4 T cells and normally represents less than 5% of this compartment (see chapter 3).
- The cytokine-driven homeostasis is differentially regulated in CD4 and CD8 T cells (816). For example, IL-15 appears to be an important regulator of the development and the maintenance of CD8, but not CD4, memory T cells.

Therefore, these important differences between the CD4 and the CD8 T cell compartments have to be taken into consideration, as it is difficult to extrapolate results obtained with one cellular compartment to the other.

5.3.3 T cell memory subsets and immune protection

5.3.3.1 Relationship between T_{CM} and T_{EM} cells

Recent conflicting experimental data have challenged our current understanding of the developmental and functional paradigm for memory T cell subsets as well as the

relationship between central and effector memory T cells. Initial *in vitro* differentiation experiments on human T cell subsets have suggested that T_{EM} may derive from T_{CM} precursors (482;487;488;817), a possibility supported by analysis of telomere lengths (482). However, using a mouse model, Ahmed and colleagues have shown that following antigen clearance, T_{EM} convert directly into T_{CM} and only then gain the ability to undergo efficient homeostatic proliferation (809). It is worth noting that, in contrast to other studies performed both in mouse (485;818) and in humans (482;487), they did not detect direct *ex vivo* effector functions among the T_{EM}, raising some doubts about the real "identity" of their T_{EM}. In fact, it is possible that the cells identified as T_{EM} by Wherry *et al.* share some phenotypic properties with real, genuine T_{EM} cells but lack the functional properties associated with this differentiation stage.

Adding to the complexity, Baron *et al.* analysed the human TCR repertoire of both T_{CM} and T_{EM} subsets and observed that their respective clonal composition markedly differs at any given time point, strongly suggesting that the genesis of these two subsets is largely independent (819). They also demonstrated that important size variations of a given clone within one subset did not lead to any change in the other subset, raising serious doubts about a possible link between the two subsets. This view is supported by *in vitro* experiments showing that cells having properties similar to those of either central or effector memory can be independently generated by controlling the stimulation conditions, notably the cytokine environment (802;820). These new data challenge the current model that stipulates that the T_{EM} subset, which contains committed progenitors with a limited lifespan, is replenished by long-lived cells from the T_{CM} pool (821;822). They favour an alternative view that CCR7 expression divides the memory T cell repertoire into two largely independent subsets, with distinct origins, possibly generated in distinct secondary lymphoid organs. These memory subsets may be generated and maintained by separate stimuli and would therefore occupy distinct homeostatic niches.

It should be noted however that these discrepancies are possibly due to the fact that these studies have been performed on different species; it is possible that there are fundamental differences between human and mouse memory T cells. For example, while a subset of

"terminally differentiated" effector memory T cells (T_{EMRA}, CD45RA+ CCR7-) is well described in humans, no markers that identify this subpopulation (if it exists) have been identified so far in the mouse. Moreover, a recent study analysing the clonal distribution of CD8 memory T cells in a murine model of influenza A infection has revealed that the clonotypic composition of the memory population residing in the lymph nodes is very similar to that of the population homing to peripheral organs such as the lung or the liver (705). Such clonotypic overlap between influenza-specific central and effector CD8 memory T cells was not observed in humans (819), providing further evidence that the regulation of memory T cell differentiation, as well as the relationship between the memory subsets, differ between mouse and human.

5.3.3.2 T_{CM} and T_{EM} in immune protection

The existence of different subsets of memory T cells raises important questions regarding the role of each subset in conferring protective immunity. The current "bi-phasic" model stipulates that T_{EM} cells, which exhibit immediate effector functions but limited proliferation, may provide a first line of defense in nonlymphoid tissue while lymphoid organ-resident T_{CM} cells, which appear to have a better proliferative potential, can give rise to a large population of effector cells that will migrate to the periphery and support the initial attack mediated by the T_{EM}.

Using two well-characterized mouse models (LCMV and LM), Wherry *et al.* showed that protective immunity is more efficiently conferred by CD8 T_{CM} cells because of their increased proliferative capacity as compared to T_{EM} (809). Adoptively transferred highly purified T_{CM} can mediate a more rapid control of viral infection than the T_{EM} subset. However, T_{EM} are more effective in reducing viremia than naïve T cells. Surprinsingly, lymph node-resident T_{CM} cells initiate a response and accumulate more rapidly at a site of peripheral challenge than nonlymphoid resident T_{EM}.

In a study aiming at understanding the ineffectiveness of killed or inactivated vaccines against intracellular pathogens, Pamer and colleagues demonstrated that vaccination with heat-killed *Listeria monocytogenes* (HKLM) leads to the expansion of memory CD8 T cells that do not mediate protective immunity, most probably because they lack immediate cytolytic activity and IFN-γ secretion capacity (823). HKLM immunization primes T cells that undergo few rounds of division without acquiring effector functions; the phenotype of these cells is similar to that of T_{CM} cells, with high expression of the lymph node homing receptor CD62L. What causes this impaired T cell differentiation following immunization with HKLM is still unclear. The authors proposed that live and killed bacteria are phagocytosed by different APCs (DCs and macrophages, respectively) and that only DCs have the capacity to promote full T cell differentiation into effector cells. It is also possible that the level of antigen presentation is much lower following priming with HKLM as compared to priming with live bacteria (because of bacterial division), leading to an incomplete/abortive CD8 T cell differentiation in HKLM-immunized mice.

This apparent discrepancy concerning the protective ability of the different memory subsets could be related to the type and the route of immunization/infection. In a recent study, it was shown that a DNA vaccine, which has been shown to generate long-term memory T cells, was effective at protecting against a high-dose systemic LCMV challenge but ineffective against a peripheral infection in solid, non-lymphoid organs (824). By contrast, mice immunized with a live virus are completely resistant to both systemic and peripheral LCMV infection. When the authors characterized the phenotype of the primed T cells, they observed that a large proportion of DNA-primed T cells did not express markers associated with effector memory differentiation as compared to T cells from mice immunized with the live virus.

Therefore, the immunological environment following exposure is then critical as it may seed different subsets of memory T cells or introduce a bias in the type of memory generated. Characteristics shown to contribute to the successful resolution of a natural infection by a specific pathogen ought to be sought in vaccine-induced responses. In a

broader perspective, it must be emphasized that besides absolute memory T cell numbers, cell phenotype and function have to be analyzed before it is reasonable to make a valid prediction concerning the *in vivo* protective capacity of a given vaccine protocol. The above should be taken in consideration in the design and evaluation of novel pathogen-customized vaccine strategies.

5.3.3.3 CD4+ cytotoxic T lymphocytes

The direct *ex vivo* detection of CD4+ T cells possessing lytic granules challenges our ordinary view of the division of labour during the immune response, with the CD8+ "killer" T cells on one hand and CD4+ "helper" T cells on the other. Results presented in chapter 3 demonstrated that perforin-expressing CD4 T cells are highly enriched in the CD45RA+CCR7- subset and that they can also be detected in a significant fraction of cells belonging to the CD45RA-CCR7- effector memory subset. The majority of these cells have lost expression of the co-stimulatory receptors CD27 and their direct *ex vivo* functional characteristics (strong IFN-γ secretion, no IL-2 production and limited proliferative capacity) resemble those of highly (or "terminally") differentiated CD8 T cells (459;487;825).

What is the role of CD4 CTLs in immune protection? It can be hypothesized that they may be directly involved in fighting infections tropic for MHC class II-expressing cells, such as HIV (activated CD4+ T cells), EBV (B cells) and *Mycobacterium tuberculosis* (macrophages). Cytotoxic CD4 T cells may play a unique role in host defense to these infections through the release of granular constituents that promote the destruction of infected APCs. Alternatively, they may lyse these cells in order to prevent an overreaction of the ongoing immune response. CD4 CTLs could be even more important to contain viruses that prevent normal expression of MHC class I molecules (such as HIV, EBV and CMV) and inhibit normal recognition and killing by CD8+ CTLs. Supporting this hypothesis, high levels of perforin-positive CD4 T cells can be detected in HIV- , EBV- and CMV-infected individuals (826;827). Moreover, two studies have

shown that Burkitt's lymphoma cells (an EBV-associated malignancy), which escape immune detection by CD8 T cells, can be destroyed by EBV-specific CD4 CTLs through MHC class II-restricted recognition (828;829).

The presence of CD4 CTLs has also been associated with other immunopathological conditions such as rheumatoid arthritis (830), Sjogren's syndrome (SS) (831), active inflammatory bowel disease (IBD) (832) and B-chronic lymphocytic leukemia (B-CLL) (833). Therefore, it is also possible that these cells do not exert specific functions during an immune response but rather are a "by-product" resulting from strong and/or chronic inflammation. Extensive proliferation and differentiation of effector CD4 T cells would then lead to the aberrant expression of cytotoxic molecules by these cells. This hypothesis is supported by experimental evidence including:

- CD4 T cell lines or clones generated by long-term *in vitro* stimulation contain a high fraction of perforin-positive cells .
- The fact that the majority of these CD4 T cells exhibit an atypical phenotype, characterized by the expression of receptors usually found on other cell types (such as KLRG1 and CD57, normally expressed by subsets of NK cells) and by the lack of expression of CD27 and CD28 co-stimulatory molecules.
- It has been demonstrated that perforin expression by T cells is regulated by DNA methylation and chromatin structure (834). It is now widely accepted that entry into cell cycle facilitates chromatin remodeling and DNA demethylation and therefore increases T cells' capacity to express effector molecules (835-838). Therefore, extensive proliferation of CD4 T cells due to persistent immune activation could facilitate accessibility to the perforin gene and lead to its "abnormal" expression.

5.3.4 Survival of the fittest: TCR affinity and selection of memory T cells

A central question concerning T cell memory generation remains: Is the selection to become memory entirely stochastic or is there a preferential selection of specific T cell clones? As it is the case for the majority of biological processes, the answer to this

question is most probably a combination of randomness and intrinsic clonal advantage. Owing to random encounters of variable duration, it is clear that T lymphocytes with intrinsic properties are activated at different time points during the immune response by stochastic exposure to APCs that present different amounts of Ag and co-stimulatory molecules, amidst various cytokines and chemokines. Experiments using TCR transgenic mouse models provide good examples of such randomness during an immune response. In such a context, since almost all T cells in these mice express the same TCR, T cell fate during and following an immune response can only be influenced by the stochastic factors described above.

However, in a normal host, each T cell clone expresses a unique TCR that has a pre-determined affinity for the Ag. As discussed earlier, TCR:ligand affinity partially dictates the strength of the signal received by the cell. It has been elegantly demonstrated by three independent groups that the capacity of T cells to proliferate and to survive *in vivo* (referred to as T cell fitness) is determined by the overall strength of the priming stimulus (674;691;839). They showed that cells receiving a weak signal are less responsive to the growth factor IL-2 due to low expression of CD25, the IL-2 receptor α-chain. These cells were also unresponsive to the homeostatic cytokines IL-7 and IL-15 and expressed low levels of the anti-apoptotic molecules Bcl-2 and Bcl-X_L. Based on these results, it can be hypothesized that high affinity T cells, because they will most likely receive a stronger TCR signal, have a certain selective advantage over low affinity cells to survive following the contraction phase of the immune response. The T cell affinity maturation process, which has been observed in some experimental models, could be a reflection of this phenomenon. As discussed earlier, the selective advantage of the high affinity T cells is probably more prominent under conditions of limited antigenic presentation.

5.3.5 Survival and persistence of memory T cells

What are the factors allowing for the survival and persistence of memory T cells? For T cells to persist as memory, two key conditions must be met. First, there must be developmental regulation for some cells to be arrested at a pluripotent differentiation

stage. Second, these pluripotent memory cells must persist for prolonged periods of time. Factors involved (or hypothesized to be involved) in memory T cell development and persistence include:

5.3.5.1 BCL6

BCL6 is a sequence-specific transcriptional repressor ubiquitously expressed in various cell types, including germinal center (GC) B cells and mature T cells. In GC B cells, it appears to confer self-renewal capacity by regulating the expression of proteins involved in the cell cycle (840). BCL6 blocks the terminal differentiation of B cells by repressing the expression of Blimp-1, a transcription factor directly involved in the development of antibody-secreting plasma cells (821;841). BCL6 can also modulate the expression of various proteins in B cells such as cytokines, chemokines as well as molecules involved in apoptosis (840;842;843). Interestingly, BCL6 expression dramatically extends the replicative lifespan of primary human B cells in culture (844). It has also been recently reported that mice lacking BCL6 have a reduced number of T cells (CD4 and CD8) with a memory phenotype and that this defect could be rescued upon expression of a BCL6 transgene under the control of a T cell-specific promoter (845). Results from adoptive transfer experiments strongly suggest that BCL6 is required for both the homeostatic proliferation and the maintenance of antigen-induced memory T cells. The role of BCL6 appears to be the stabilization of a subset of T cells at a particular differentiation state, conferring them with stem cell-like function. A homologue of BCL6, BCL6-associated zinc finger protein (BAZF) is expressed in activated splenocytes and could be another candidate (846).

5.3.5.2 LKLF

An interesting candidate that could confer memory properties to a subset of T cells is the lung Krüppel-like transcription factor (LKLF). LKLF is a member of the Krüppel-like factor (KLF) family of zinc-finger transcription factors (198). It has been shown that members of this family regulate the terminal differentiation of multiple cell lineages, including erythrocytes, keratinocytes and vascular endothelial cells (847). The expression

of LKLF is tightly regulated during T cell development. Mature single-positive T cells express high levels of LKLF that regulates their quiescence and survival (848). Following T cell activation, the expression of LKLF is rapidly down-regulated and is only reinduced at high levels in "quiescent" memory T cells (849;850). Splenic and lymph node T cells from LKLF-deficient mice display an abnormal activated phenotype and undergo an apoptotic process similar to AICD (848). Overexpression of LKLF in Jurkat T cells leads to inhibition of their proliferation and to acquisition of a quiescent T cell phenotype by a mechanism involving blockade of a c-Myc-dependent pathway (847). LKLF expression in memory T cells is partly regulated by the presence of cytokines such as IL-2 and IL-7 following T cell activation. Importantly, it appears that the survival of memory T cells is not due to the presence of a subpopulation of activated T cells that did not down-regulate LKLF expression but rather by cytokine-induced reexpression of LKLF by a subset of activated T cells (849). These results suggest that reexpression of LKLF following T cell activation blocks the differentiation of T cells, prevents cell death and favours their development into long-lived memory T cells.

5.3.5.3 Bcl-2 family

Because they are protected from the massive death of activated T cells that occurs following a normal immune response, it has been hypothesized that memory T cells express high levels of anti-apoptotic proteins. Members of the Bcl-2 family include anti-apoptotic molecules that promote the survival of memory T cells. Transgenic mice that constitutively express Bcl-2 in T cells show progressive accumulation of thymocytes and mature T cells, suggesting a role for this molecule in promoting T cell survival *in vivo* (851;852). Bcl-$2^{-/-}$ mice lose all T cells as they age (853). It has been shown that following a response to a viral infection, memory CD8+ T cells express high levels of Bcl-2 compared to naïve and effectors cells (850;854). The recognized survival effects of IL-7 are due, in part, to the ability of IL-7 to increase and/or sustain Bcl-2 expression in T lymphocytes (855;856). Interestingly, the upregulation of Bcl-2 in T cells following the contraction phase of the immune response correlates with the re-expression of the IL-7 receptor α (IL-7Rα) (816). In CD4+ T cells, CD134 co-stimulation has been shown to

promote expression of Bcl-X_L and Bcl-2 and to be essential for long-term T cell survival (857). In fact, T cell fate appears to be predictable from the ratios between pro- and anti- apoptotic molecules of the Bcl-2 family (452).

5.3.5.4 CaMKII

A protein that has been shown to promote accumulation of T lymphocytes with a memory phenotype is calcium/calmodulin kinase II (CaMKII) (858). This kinase family mediates part of the cellular response to calcium in different tissues. Interestingly, in neurones, this enzyme is believed to be involved in the long-term synaptic memory (859). Given the similiraties between the neurological and the immunological systems, especially at the level of the synapse (860;861), it is tempting to make a parallel between immunological and neuronal memory formation. Using transgenic mice expressing a calcium-independent (constitutively active) CaMKII, Hedrick and coworkers observed that these mice exhibited a 1.3- to 2-fold increase in the number of memory T cells in the peripheral lymphoid organs. They showed that T lymphocytes from the transgenic mice are less prone to die following *in vitro* antigen stimulation compared to cells from wild-type mice. Interestingly, it seems that activated, dividing T cells from wild-type mice acquire autonomous (Ca^{2+}-independent) CaMKII activity after 4-5 days in culture.

As previously discussed, it is well established that the extent of calcium influx can be greatly modulated by the strength of T cell activation (218;862) and can influence its outcome by regulating several signalling pathways (863). Consequently, it is possible that memory T lymphocytes may be preferentially selected to survive on the basis of a high magnitude and sustained Ca^{2+} response leading to sustained activation and autonomous activity of CaMKII. However, because memory T cells can survive for several years, it seems unlikely that autonomous CaMKII activity can be maintained in T lymphocytes for a such prolonged period of time. Moreover, the possible link between CaMKII and T cell memory appears rather controversial and awaits further confirmation; no scientific papers related to this topic have been published since the original publication by Bui *et al.* in 2000.

5.3.5.5 Cytokines

Beyond direct expression of specific genes, several groups have highlighted the important regulatory role of soluble mediators in the generation and maintenance of memory T cells. Cytokines have pleiotropic effects on T cells: IL-2, IL-7, IL-15 and IL-21 are now under much scrutiny (816). The receptors for these cytokines have the particularity of using the common γ chain. The functional receptors for IL-2 and IL-15 also share the β chain, fine specificity being defined by the further expression of private α chains. Signalling from the IL-2R and IL-15R has so far only been associated with the β and γ chain components they share. In spite of this, engagement of these receptors leads to different outcomes. In fact, while IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ mice suffer from lymphadenopathy and autoimmunity, IL-$15R\alpha^{-/-}$ mice display lymphopenia and a selective reduction of in the number of $CD8^+$ T cells with a memory phenotype. IL-2 has been linked to T cell survival and proliferative expansion early in an immune response, however IL-2 is also required later on for triggering the death pathway (436). IL-$2^{-/-}$ mice and IL-$2R\alpha^{-/-}$ mice show accumulations of activated T cells associated with reduced AICD (439;441;864;865). In contrast, both IL-$15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice show a 2-3 fold decrease in the number of CD8 T cells with a $CD44^{hi}$ memory phenotype (866;867). Moreover, IL-15 transgenic mice have 3 times more memory cells compared to syngeneic wild-type mice (868;869). In fact, using combinations of blocking antibodies specific for IL-2, IL-2Rα and IL-2Rβ, Ku *et al.* demonstrated that, whereas IL-15 drives the slow proliferation of memory $CD8^+$ T cells *in vivo*, this proliferation is increased by the inhibition of IL-2 (870). This increase is attributed both to an enhanced proliferation of the memory cells and to reduced AICD. Finally, IL-2 and IL-15 have been shown to differentially modulate both the recruitment and maturation of memory CD8 T lymphocytes, as previously discussed (802).

Another cytokine, IL-7, appears to be necessary for the establishment of T cell memory and may play a role in the maintenance of memory T cell homeostasis (871;872).

Blocking of IL-7 and IL-7R appeared to have only a modest effect on memory $CD8^+$ T cell proliferation (870). Nevertheless, IL-7 is also linked to memory T cell homeostasis. $IL\text{-}7R^{-/-}$ CD8 T cells transferred into non-irradiated wild-type recipient mice were shown to proliferate at levels comparable to that of wild-type cells following subsequent *in vivo* stimulation. However, the survival of the $IL\text{-}7R^{-/-}$ CD8 T cells was greatly impaired as compared to their wild-type counterpart as the former failed to persist in large numbers (871). Interestingly, this impairment in survival of the $IL\text{-}7^{-/-}$ CD8 T cells was correlated with a failure to re-express Bcl-2 to normal levels following the peak of proliferation associated with the antigen-driven response (871).

Therefore, it is clear that the presence of specific cytokines in the immediate environment has the potential to influence the fate of the responding T cells. However, evidence is now accumulating that it is the ability of a given T cell to respond to these cytokines through the timely expression of the appropriate receptors, both during and after the contraction phase of the immune response, that will primarily dictate whether this T cell dies or develops and persists as memory.

5.4 Future directions and conclusion

The following reflections and proposed experimental approaches may also be of interest in order to guide future forays in the characterization of the mechanisms underlying T cell repertoire expansion and memory T lymphocyte development, differentiation and persistence.

1. Given the evidence that memory T cell precursors are already present at the peak of the primary immune response, what are the phenotypical and functional properties of these precursors? To resolve this issue, it would be necessary to develop an experimental model that would allow the specific identification of the memory T cell precursors that are present among the large pool of effector T

cells. An original transgenic mouse model has been developed by Jacob and Baltimore in 1999 (790), but the irrevocable evidence that their experimental system permits the specific labelling of memory T lymphocytes precursors still awaits further confirmation. Using this transgenic system, it would then be possible by multi-parametric flow cytometry to find a combination of markers that identify (or at least that significantly enrich) the memory T cell precursors. These phenotypic markers could be used in normal, non-transgenic murine models, to study the development of memory T cells in various infections. Moreover, the characterization of the functional properties associated with memory "potential" could be assessed by comparing, at different time points, the gene expression profile of the memory precursors with that of other T cell subsets (naïve, effector and memory) using a DNA microarray approach (804).

2. Are antigen-specific T cells selected to mature differentially into the various memory T cell subsets on the basis of the affinity of their TCR for their ligands? This interesting question could be assessed experimentally by using an *in vivo* system making use of superantigens, similar to the *in vitro* model described in chapter 2. By evaluating the distribution of different TCRVβ-bearing T cells among the central and effector memory subsets following immunization with a superantigen, it would be possible to determine if the maturation of memory T cells is mainly stochastic (all SAgs-reactive TCRVβs randomly distributed between the different subsets) or driven by a TCR affinity-based mechanism (preferential distribution of some TCRVβs in specific memory subsets). Alternatively, this could be assessed in antigen-restricted systems by a tetramer dissociation assay that has already been used by several groups (169;701;702;873). This assay determines the overall TCR affinity of a heterogeneous population of T cells by monitoring the kinetics with which a fluorochrome-labelled MHC tetramers dissociate from the TCR complex. The net result is that the rate at which antigen-specific tetramer labelling is gradually lost from the various T cells of the population correlates with the overall TCR affinity of this population for the antigenic complex. Therefore, by comparing

the tetramer dissociation rate between the different memory T cell subsets, it would be possible to determine if a TCR affinity-based skewing of the repertoire takes place during an infection/immunization.

3. What are the factors controlling the differentiation of T cells into T_{CM}, T_{EM} and T_{EMRA}? This could be assessed experimentally by varying different parameters in the context of vaccination trials. Paramaters that could be tested include:
    - the route of immunization (intravenous, intramuscular, etc.);
    - the nature of the immunogen (live, attenuated or inactivated pathogen, Ag-loaded DCs, protein, DNA, peptides);
    - the amount of immunogen used for vaccination (i.e. the antigen load);
    - the nature of the adjuvant;
    - the addition of potential immune "boosters" (co-stimulatory molecules, cytokines) during and/or after immunization.

    Importantly, the identification of the parameters that bias the development of memory T cells toward a given subset in conjunction with the determination of the "optimal" memory T cell distribution that confers immune protection against infectious pathogens could allow the development of very effective vaccination protocols.

Overall, the studies presented in this thesis provide novel insight into the dynamics of T cell clonal expansion as well as phenotypic and functional attributes of memory CD4 T lymphocytes. Our characterization of the clonotypic and functional diversity of the immune response provides us with a more complete understanding of T cell-mediated immunity. Elucidation of the fundamental phenotypic and functional features of T cell subsets is critical to deciphering the multifaceted nature of the T cell-mediated immune response. Also, a better knowledge of the mechanisms by which successful and long-lasting cellular immune responses are generated will undoubtedly facilitate the optimization of effective vaccines for preventing infectious diseases, as well as for fighting chronic infections and tumours.

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Appendix