Cell-cell interactions *in situ* during the early phase of T cell priming

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"We shall not cease from exploration, and the end of all our exploring will be to arrive where we started, and know the place for the very first time."

(TS Eliot, 1888-1965)

to my parents

Abstract

Cell-cell interactions *in situ* during the early phase of T cell priming

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Successful induction of an adaptive immune response depends on antigen dose, the duration of antigen presentation and the cellular environment in which antigen is presented. These parameters may vary according to the particular epitope recognized by T- or B-cells. However, the *in situ* behaviour of clonal or polyclonal T cell populations during the immune response has only recently become amenable to precise experimental analysis. Here, we isolated and characterized T cell/APC clusters as a functional compartment of initial T cell proliferation. This way, it has been possible to bridge existing *in vitro* and *in situ* studies.

Cluster formation was found to occur independent of specific antigen application and included segregation of recent lymph node immigrants (measured as CD62L down-regulation). Furthermore, initiation of T cell proliferation could be attributed to clusters and was followed by cell-cell contact-independent division at later stages of clonal expansion. This finding was confirmed in six different transgenic models including both T cell lineages (CD4, CD8) as well as two different MHC backgrounds ($H2^b$, $H2^d$). Interestingly, even though all transferred T cell populations could be localized in clusters at the same time, completion of the first round of cell division of T lymphocytes specific for a subdominant epitope and a weak agonist lagged behind that of cells specific for dominant epitopes. The T cell wave needed approximately 32-36 hours to pass through the cluster 'compartment'. These results concomitant with current literature provide a basis to combine and extend current ideas in a revised model of T cell activation.

In addition, the hCRP model allowed addressing the mutual influence of T cell populations specific for epitopes of the same protein. Using the method of cluster isolation, it could be shown that both populations recognize their antigen on the same APC – an important prerequisite for events such as linked help or suppression. Linked help towards the subdominant epitope could be observed in a situation in which antigen was transiently available but not under prolonged transient antigen availability.

Abstract (German)

Zell-Zellinteraktionen *in situ* während der frühen Phase der T-Zellaktivierung

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Die erfolgreiche Induktion einer adaptiven Immunantwort ist von der Antigendosis, der Dauer der Antigenpräsentation sowie der zellulären Umgebung, in der das Antigen präsentiert wird, abhängig. Diese Parameter können je nach Epitop, das von T- oder B-Zellen erkannt wird, variieren. Das *in situ* Verhalten klonaler oder polyklonaler T-Zellpopulationen im Verlauf einer Immunantwort ist erst in den letzten Jahren einer eingehenderen Untersuchung zugänglich geworden. In dieser Arbeit wird die Isolation und Chrakterisierung von T-Zell/APZ-Clustern als funktionelle Einheit der initialen T-Zellantwort beschrieben.

Die Clusterbildung fand unabhängig von spezifischer Antigengabe statt und beinhaltete die Segregation von Zellen, die erst kürzlich in den Lymphknoten eingewandert waren (gemessen als Abnahme der Oberflächenexpression von CD62L). Desweiteren konnte die Initiation der T-Zellteilung Clustern zugeschrieben werden und ging zu einem späteren Zeitpunkt in eine vom DZ-Kontakt unabhängige Proliferation über. Diese Beobachtung konnte in sechs verschiedenen transgenen Modellen bestätigt werden, die sowohl beide T-Zelllinien (CD4, CD8) als auch zwei verschiedene MHC Haplotypen ($H2^b$, $H2^d$) einschlossen. Obwohl T-Zellen aller Modelle zur gleichen Zeit in Clustern lokalisiert werden konnten, benötigten T-Lymphozyten, die ein subdominantes Epitop und einen schwachen Agonisten erkennen, länger, um ihre erste Zellteilung zu vollenden, als Zellen die spezifisch auf ein dominantes Epitop reagierten. Die ‚T-Zell-Welle' durchlief das Cluster-Kompartment in etwa 32-36 Stunden. Diese Resultate stimmen mit aktueller Literatur überein und bieten einen Ansatz, derzeitig diskutierte Modelle miteinander zu verbinden und zu einem revidierten Modell der T-Zellaktivierung zu erweitern.

Darüberhinaus erlaubt das hCRP-Modell, den gegenseitigen Einfluß zweier T-Zellpopulationen, die Epitope desselben Proteins erkennen, zu untersuchen. Mit Hilfe der Clusterisolation konnte gezeigt werden, daß beide Populationen ihr Antigen auf derselben APZ erkennen – eine wichtige Voraussetzung für 'linked help' oder 'linked suppression'. Unter Bedingungen, in denen Antigen nur über sehr kurze Zeit verfügbar ist, konnte 'linked help' für Zellen, die gegen das subdominante Epitop gerichtet sind, gezeigt werden. Dieser Einfluß ging verloren, sobald das Antigen über etwas längere Zeit vorhanden war.

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Introduction

1 Basic Concepts in Immunology

In higher organisms the immune system can be divided into two functional units, namely the *innate* (natural) and the *adaptive* (acquired) one, both working closely together. Innate immunity provides the first barrier for infections, eliminating most of the pathogens before serious damage can occur. It is mainly non-inducible and displays neither (or only partial) specificity nor memory functions. Therefore susceptibility remains the same even after repeated infections. The components of the innate immunity are of both soluble (lysozyme, complement, acute phase proteins such as C-reactive protein (CRP) and interferons) and cellular nature (macrophages, neutrophils and natural killer cells (NK cells). In case a pathogen manages to slip through this line of defense, the adaptive response takes over. Due to the work of Pasteur, Koch, Metchnikoff and other microbiologists, its characteristic features were identified: firstly, it is inducible i.e. the system responds specifically to antigens. Secondly, the most striking feature is the generation of an immunological memory, leading to the accurate recognition of an antigen upon re-infection. Quite often an adaptive immune response provides life long protective immunity (Golub and Green, 1991). There are several mechanisms working together in such a response, and they involve *humoral* as well as *cellular* components. Families of antigen binding molecules that are expressed on or produced by lymphocytes determine specificity, diversity and memory: immunoglobulins (antibodies) and T cell receptors (TCRs). Cellular immunity involves the activity of inflammatory T helper lymphocytes, which are able to lyse infected cells upon recognition of foreign antigens presented on their surfaces. The humoral immune response is based on specific antigen-binding soluble factors called *antibodies* that are produced by B lymphocytes. Due to the nature of their development, it was long thought that each B- and T cell is restricted to recognize a single antigen but this has been proven not to be true (Mason, 1998). As a population the cells of the immune system possess millions of specificities (the theoretical diversity of the pre-selection repertoire mouse or human αβ T cell repertoire, i.e. before its shaping by selection for self-MHC-recognition and avidity, has been estimated at about 10^{14}, Davis and Bjorkman, 1988). A population of resting T cells is activated only after specific antigenic stimulation leading to one of the fundamental features of immune responses:

proliferation. This process is called *clonal selection* (Burnet, 1959) and the basic premises of this theory are (paraphrased from Burnet, 1969):

- existence of cells differentiated for immune function being evident by their ability to react with specific antigen,
- determination of the antibody specificity by the genetic endowment of the cell producing it,
- sharp limitation of the range of immune reactivity of any lymphocyte by a process of phenotypic restriction,
- origin of the diversity of immune specificity having to be sought at the genetic level,
- antigen acting essentially as a signal or stimulus to such cells that are competent by expressing specific receptors to it.

Under the influence of T cells and monocytes, B cells differentiate into antibody secreting plasma cells and memory cells.
All cells involved in immune responses are sequestered in lymphoid and non-lymphoid organs or circulate through the body. Primary lymphoid organs are important for the differentiation of pluripotent stem cells into T- (thymus) and B cells (Bursa Fabricius) whereas secondary lymphoid organs such as lymph nodes, spleen and lymphatic tissues associated with mucosa (Peyer's patches, gut, lungs and tonsils) provide sites for the initiation of an adaptive immune response. Different lymphocytes inhabit different regions of these organs and trafficking is mediated by homing receptors on their cell surface (Janeway and Travers, 2001).

2 T cell Biology

2.1 Basic Concepts of T cell Activation

2.1.1 The T cell Receptor

T cells are the main carrier of cell-mediated immunity and have an important regulatory function in humoral immune responses. As mentioned above, specificity is provided by the cell surface expression of receptors. Each mature T cell bears approximately 40,000 T cell receptor molecules (Meuer et al., 1982), which are composed of two disulfide-linked, glycosylated polypeptide chains forming a heterodimer. The vast majority of mature

lymphocytes in mice express receptors composed of α- and β-chains, only very few bear the alternative type of a γδ heterodimer (2-5% of murine peripheral T cells): While the former clearly mediates antigen recognition, the function of the latter is about to be fully elucidated (Schild et al., 1994). The T cell receptor displays homology to a membrane-bound fragment of immunoglobulins, and therefore it is not surprising that B- and T cells recruit the same mechanisms operating on homologous genes to generate these molecules. Analogous to the heavy and light chains of the B cell receptor, the formation of mature antigen receptors on T cells is a result of somatic recombination of separate gene segments. In α-chains, one of multiple V-(*variable*) elements is coupled to an adjacent J-(*joining*) element, which in turn is joined to the only exon encoding the C-(*constant*) region. In β-chains an additional D-(*diversity*) segment is included to give V(D)J exons, which then are also joined to a C-element. While there is only one C_α gene, two C_β genes are known. Nevertheless, no functional difference between their products could be identified. This so-called *TCR-rearrangement* requires mechanisms acting very precisely (but still not perfect) to ensure only corresponding gene segments are joined to each other: recombination signal sequences (RSS) determine the site of cleavage, which is performed by proteins that introduce double strand breaks. Some of these proteins are encoded by recombination activating genes (RAG1 and RAG2). Improper joining of the segments, i.e. by the addition of P-(palindromic) and N-(non-templated) nucleotides increases TCR diversity. The actual variability is concentrated in the so-called *complementarity determining regions* (CDRs), with CDR1 and CDR2 being encoded within the V segments themselves, while CDR3 is located at the position of the P- and N-nucleotides. Unlike B cells, T cells do not extend receptor diversity by somatic hypermutation upon antigen contact. Most T cells are restricted to the production of a TCR of only a single structure even though they actually have the potential of producing two different ones (about one third of the T cell population expresses two α-chains (Padovan et al., 1993)). This process of making use of only one of the two chromosomes is referred to as *allelic exclusion*. The cytoplasmatic domain of the TCR is rather short and thus lacks sufficient signaling capacity. It is non-covalently associated with a complex of invariant proteins, collectively termed CD3 complex, which are involved in signal transduction.

2.1.2 Antigen Recognition

In contrast to B cells and antibodies, T cells are not able to recognize antigen in its native form but depend on peptide fragments that are presented by molecules of the major

histocompatibility complex (MHC) on the surface of antigen presenting cells (APC) (Cresswell and Howard, 1997). The specificity of a TCR for a given MHC allelic product is referred to as *MHC restriction*.

The discovery of the MHC was based on it encoding transplantation antigens inducing rejection of tissue transferred from one individual to another (Gorer et al., 1936). The MHC is, in fact, one of the most highly polymorphic gene complexes known and is not only multi-allelic, but also multigenic. The alleles are numerous, divergent, and evenly distributed within populations (Parham and Ohta, 1996). MHC class I molecules are expressed constitutively on most somatic nucleated cells or, if not, can be induced by lymphokines such as interferon γ. They present endogenous self-proteins and viral proteins produced by the cell's biosynthetical machinery in the cytosol (Janeway and Travers, 2001). The expression of MHC class II molecules is restricted to professional antigen presenting cells such as dendritic cells (DC), B cells, activated macrophages (Mϕ), cortical (cTEC) and medullary (mTEC) epithelial cells of the thymus (Hämmerling and McDevitt, 1974; Hämmerling et al., 1975; Rouse and Weissman, 1982). MHC class II molecules mainly present peptides derived from proteins that have been endocytosed and processed by APC. There is evidence that exogenous antigens are also presented on MHC class I molecules (Kurts et al., 1997; Rodriguez et al., 1999). This mechanism is referred to as '*cross-presentation'*.

Specific antigen recognition is based on a tetramolecular complex of MHC molecule, presented peptide, TCR and one of its co-receptors, either CD4 or CD8. These co-receptors bind to nonpolymorphic regions of MHC molecules. They increase the avidity of a T cell to its cognate MHC/antigen-complex and they are also involved in intracellular signaling cascades. $CD4^+$ T cells can only recognize antigen fragments bound to MHC class II molecules, whereas $CD8^+$ and inflammatory T helper lymphocytes are restricted to peptides presented by class I molecules (Zinkernagel and Doherty, 1974, 1979, 1997). Recognition of MHC-peptide complexes results in different T cell effector functions: either killing of the infected cells (CD8 T cells, cytotoxic T helper cells) or enhancement of cellular and humoral responses (CD4 T cells).

2.1.3 Dendritic Cells as Professional Antigen-presenters

Dendritic cells play a key role in the immune system: They are the only antigen-presenting cells able to activate naive T cells to take part in primary immune responses and they can also stimulate secondary responses of memory cells (as do macrophages and B cells) so that they

become effector cells. Thus, DC initiate adaptive immune responses and, depending on their maturation status, may also determine tolerance.

Dendritic cells represent a heterogeneous population of cells. The majority of DC develops from myeloid stem cell precursors in the bone marrow (Steinmann et al., 1991; Inaba et al., 1993) and resides at low numbers throughout all non-lymphatic tissues in a so-called 'immature' state. Immature DC, e.g. Langerhans cells (LC) in the skin, retain large pools of MHC molecules intracellularly but express only few on the surface and are negative for co-stimulatory molecules such as B7.1 and B7.2. Furthermore, they are characterized by their ability to take up and process pathogens and antigen (Romani et al., 1989) but having only limited capacity to stimulate naive T cells. DC sample their environment by constitutive micropinocytosis of extracellular fluid, and they also express different receptors mediating endocytosis and phagocytosis of pathogens and antigens. In response to inflammatory mediators (such as TNF-α, IL-1β), cytokines or necrotic cells, immature DC migrate to the T cell areas of secondary lymphoid tissues via afferent lymphatic vessels (Cumberbatch and Kimber, 1992; Cumberbatch et al., 1997). Here they undergo a switch from antigen capturing to antigen presenting and thus T cell-stimulating cells. This process, defined as *DC maturation*, includes assembly of MHC class I and class II complexes, up-regulation of co-stimulatory molecules and secretion of cytokines. In addition, with maturation, DC rearrange their cytoskeleton and increase their mobility. They 'convert' to effective stimulators of naive as well as resting 'memory' T cells (Randolph et al., 1999).

While immature DC very efficiently capture exogenous antigens and, upon maturation, load the antigenic peptides onto preformed empty and newly synthesized MHC class II molecules, mature DC shut off antigen capture and class II synthesis, and accumulate the newly formed complexes on the cell surface. The complexes acquire long half-lives – up to 100 hours in vitro –, allowing the DC to retain them for several days. Thereby presentation of antigens associated with infection is maximized. On the other hand, MHC class I-peptide complexes have much shorter half-lives (about 10 hours) and their presentation has to be sustained by continuous synthesis and loading (Lanzavecchia and Sallusto, 2000).

DC are probably the most relevant source of IL-12, the major cytokine driving Th1 polarization (see 2.2; Trinchieri, 1998). IL-12 production by DC is limited to a narrow time frame following stimulation and appears to be tightly regulated (Langenkamp et al., 2000). Thus, availability of IL-12 has to be maintained by a continuous immigration of recently activated DC. This mechanism allows the immune system to balance a Th1 response with a subsequent Th2 response even in case of the presence of pathogens strongly inducing IL-12.

In addition, DC produce IL-18 and IFN-α, sometimes IL-4 and IL-10, which downregulates their own function as well as T cell responses.
Mature DC have a finite life expectancy, affecting their capability to stimulate T cells. There is evidence that once they have reached the lymph node, they survive only a few days and disappear within 48 hours after interacting with antigen-specific T cells (Ingulli, 1997; Kurts, 1996).
Apart from the 'classical' myeloid-derived DC, a second type of DC can be found in thymus, spleen and lymph node: cells, which are derived from lymphoid precursors (Crowley et al., 1989; Wu et al., 1991). In contrast to the myeloid lineage they express a CD8α-chain. Despite their phenotypic differences both populations behave similarly in processing and presenting antigens (Dalloul et al., 1999), but the outcome of T cell activation is different: while $CD8\alpha^-$ dendritic cells preferentially induce Th2-type responses, $CD8\alpha^+$ cells induce responses of the Th1-type (Heufler et al. 1996; Stumbles et al., 1998). Recently it could be shown that the $CD8^+$- but not the $CD8^-$ subset is involved in cross presentation (den Haan et al., 2000).

2.1.4 T cell Activation

For efficient activation of naive and resting T cells sole MHC-recognition alone is usually not sufficient and appropriate co-stimulation is required (2-signal model). MHC-peptide-T cell interactions have to be sustained and interactions of weak affinities have to be 'supported', i.e. enhanced. Compensation is carried out via additional co-stimulatory receptor/ligand molecules.
Physiological T cell activation probably includes cytoskeletal polarization as well as large-scale redistribution of cell surface molecules to give *supramolecular activation clusters* (SMACs; Monks et al., 1998). Thus, an organized contact interface is formed between T cell and APC. Because of many similarities with neuronal synapses, this cell-cell junction was coined *immunological synapse* (IS; Paul and Seder, 1994). While the duration of signaling is in the order of hours, the activating and inhibitory molecular interactions in the IS have half-lives in the order of seconds. The mature synapse consists of a central cluster of T cell receptors surrounded by a ring of adhesion molecules (Monks et al., 1998). Its formation can be broken down into several steps (van der Merwe et al., 2000; Dustin and Chan, 2000; Dustin and Cooper, 2000):

Polarization and attraction to potential APC by chemoattractants. A necessary factor in T cell activation is the encounter of a T cell with an antigen-presenting cell, such as a DC.

Circulating T cells are triggered by chemokines (e.g. secondary lymphoid tissue chemokine) to pass through the high endothelial venules of blood vessels and enter the T cell areas of lymph nodes. At the same time, activated DC are also attracted to migrate from the tissue of infection to lymphatic organs where they are able to come in proximity and thus interact with many T cells. The initial reaction induced by a chemokine binding to its receptor includes the cytoskeletal reorganization of the T cell. This takes place even before the T cell makes contact with the APC and is a crucial preparation for antigen recognition (Dustin and Chan, review, 2000).

Non-antigen-specific adhesion. Adhesion requires an interaction of sufficient strength. The abundance of appropriate MHC-peptide complexes is too low, and their size too small (7 nm) to function as initial adhesion mediators. Therefore, adhesion molecules of some other kind are required. Several classes of such receptors appear to be important for T cell/APC interaction: the integrin family members LFA-1 (interacting with ICAM-1) and VLA-4 (interacting with VCAM-1), and the smaller immunoglobulin superfamily molecules CD2 (interacting with CD48), and CD28 (interacting with CD80 or CD86). They are larger (14-15 nm), abundantly present on T cells and form the so-called peripheral SMACs.
The interaction of adhesion receptors with their ligands is of low affinity. It is therefore important to achieve an appropriate alignment of the apposing membranes to obtain a strong 'two-dimensional' affinity (the physiological affinity of a given receptor as unit of surface density). An important aspect that should be mentioned is the fact that adhesion receptor affinity is regulated by TCR (Dustin and Springer, 1989). To date, this mechanism of positive feedback has not been fully understood.

Engagement of TCR by MHC-peptide. At early time-points of synapse formation, junction formation includes engagement of MHC-peptide complexes in the outer ring, which are transported to the inner cluster area at later stages.
TCR engagement is remarkably sensitive and flexible: Its affinities are in the range of 10^{-6}M and it has half-lives in the range of seconds (for foreign peptide-MHC, Matsui et al, 1994). Very few foreign MHC-peptide complexes are necessary to trigger proliferation and synapse formation. It could be shown that full T cell activation correlated with synapse formation with a threshold density of ≥ 60 MHC-peptide complexes per square micrometer. Furthermore, 0.2 molecules per square micrometer turned out to be the equivalent of 100-200 MHC-peptide complexes per APC, the minimum number of agonist complexes needed to provide sufficient stimulation for IL-2 production by T cells (Grakoui et al., 1999).

Synapse formation. TCR/MHC-peptide engagement results in assembly of signaling complexes, generation of second messengers (e.g. Src-, Syk- and Tec-families of protein kinases) and induction of cytoskeletal changes. These include polymerization of actin at the interface of T cell and APC as well as reorientation of the microtubuli-organizing center (MTOC). Reorientation of the MTOC is an indicator for Golgi-apparatus-movement by which constitutive and regulated secretion are directed to the IS. Both, actin polymerization and MTOC reorientation are required to halt T cell migration. This is a critical factor for *in vivo* responses (Ingulli, 1997). Another molecule that is involved in the process of stopping migration is the co-receptor CD4 (Grakoui et al., 1999).

TCR transportation from the initial site of engagement to the center of synapse is a remarkably active one, and may last up to 5-10 min until formation of the central SMAC (cSMAC) is complete. The central SMAC includes TCR/CD3, peptide-MHC, CD28/CD80, and cytoplasmatic signaling molecules (e.g. lck, fyn) and the synapse is now called *mature*. Interestingly, central cluster MHC-peptide complexes are not exchanged with free MHC-peptide from the outside (photobleaching recovery experiments; Grakoui et al., 1999).

The redistribution of cell-surface molecules has implicated two distinct transport processes: polarized secretion of cytoplasmatic storage vesicles, and redistribution of molecules already at the surface.

Stabilization. Another critical parameter for T cell activation is the duration of signaling. Maintenance of IS over many hours may be promoted by molecular changes during maturation of the synapse. The localization of adhesion molecules may play a role in synapse stabilization: CD28/CD80 are frequently clustered in the center of the synapse, while CD2/CD48 often accumulate in outer rings.

Alternatively, stabilization of the synapse could be promoted by the recruitment of so-called *lipid rafts*. Lipid rafts are microdomains in the plasmamembrane of T cells (and many other cells as well), which are rich in glycosphingolipids and cholesterol. Certain cell-surface molecules such as CD48 and CD4 are preferentially associated with these structures, while others, notably CD45, are not. Thus, lipid rafts provide a microenvironment in which signaling molecules necessary for T cell activation are readily clustered. Recent data describe the *active* reorganization of lipid rafts to the site of T cell activation. Binding of the associated molecules to ligands on an APC could provide a *passive* mechanism or the accumulation of rafts at the T cell/APC interface. Lipid rafts appear to play an important role in T cell triggering as their chemical disruption or mutations abrogate T cell activation.

Repolarization (Termination).

Cell-cell interaction has been described to occur between 24-48 hours in vivo (Ingulli, 1997). The organization of the synapse is a crucial factor determining the length of T cell/APC interaction: Poor organization results in spontaneous termination within a time frame of 30 min. The T cell moves away from the site of interaction, and the cSMAC is degraded or shed. The T cell may now acquire a new polarity.

2.2 Th1 and Th2: Subsets of $CD4^+$ Helper Cells

Activated $CD4^+$ T lymphocytes can be classified into two distinct populations based on their cytokine profiles and on their effector functions: T helper 1 (Th1) and T helper 2 (Th2). This conveniently subdivides T cell immune responses into those specialized for defense against intracellular pathogens including viruses and some bacteria (Th1), and a second for defense against large extracellular pathogens such as helminths (Th2). Each subset produces cytokines that serve as autocrine growth and differentiation factors. The cytokine production of the two subsets also cross-regulates each other's development and activity. Mosmann et al. (1986) were able to show that Th1 cells secrete IL-2, IFN-γ, TNF-α and TNF-β, cytokines, which are usually associated with inflammatory responses. IFN-γ induces the switching of B lymphocytes to certain IgG isotypes and activates macrophages enhancing their microbicidal actions. The principal function is to elicit phagocyte-mediated defense against infection. Some Th1 cells acquire cytolytic capacity. Both, IL-2 and IFN-γ promote the differentiation of $CD8^+$ lymphocytes into active cytotoxic cells. On the other hand, the Th2 subset produces cytokines such as IL-4, -5, -6, -10, and 13, with IL-4 being the major inducer of IgG_1 and IgE responses, and IL-5 being the principal eosinophil-activating cytokine (Coffman et al., 1988).

To date, it has not been possible to find a complete explanation for the stability of Th1/Th2 populations, which, to a large extend, may be influenced by the conditions of initial priming. Apart from the cytokine patterns they secrete, their effector functions, and their activation requirements, the heterogeneity of Th subsets extends as well to their susceptibility to activation-induced cell death (AICD). Being a mechanism for peripheral depletion of $CD4^+$ cells, AICD is implicated in the loss of effector cells, and thus in the termination of immune responses. Quite often an imbalance in Th1 and Th2 populations can be observed and Th1 effectors are more susceptible to AICD than are Th2 effectors (Zhang et al., 1997).

2.3 Development and Tolerance of T lymphocytes

The immune system has to cope with the enormous variety of antigens its host may encounter during life. This difficulty is partly solved by the generation of a large repertoire of T lymphocytes, each bearing a different receptor. As the receptor diversity is generated by a mechanism that acts 'randomly' on the various gene segments, self-reactive antigen receptors are likely to be produced as well. How does the immune system tolerate the presence of these potentially destructive T cells? About half a century ago, Owen et al. (1946) were the first to discover that 'tolerance' of self-components is not inherited but acquired. Many genes that are involved in the regulation of the developmental stages have been identified, and it is now known that the T cells are 'educated' to distinguish self from non-self by two processes termed *positive* and *negative selection*. Together they result in a repertoire that is MHC-restricted and almost fully tolerant to self, but the details of both mechanisms are still far from understood (Benoist et al., 1997; Marrack et al., 1997).

Thymic 'Education' (Positive and Negative Selection)

The developing T cell repertoire is shaped by two mechanisms taking place in the thymus: *Positive selection* of T cells describes a process that depends on the interaction of TCR with MHC molecules on cortical thymic epithelia, and is an essential step for differentiation of immature thymocytes into mature self-MHC-restricted cells. *Negative selection* ensures elimination of potentially autoaggressive T cells. According to the widely accepted *model of affinity/avidity*, an intermediate affinity/avidity of the TCR to the MHC is necessary for the immature thymocyte to be rescued from programmed cell death. Whereas no signaling results in death by neglect, cells with too strong affinity/avidity interactions are subject to apoptosis (Ashton-Rickardt et al., 1994; Nossal et al., 1994). Only those thymocytes with intermediate signaling strength are positively selected, and thus allowed to either continue maturation or become subject to negative selection.

The thymus is made up of three physically distinct areas: an outer subcapsular zone, the cortex, and the medulla. Pre-T lymphocytes initially develop from bone marrow stem cells and then migrate to the subcapsular zone. When migrating into the cortex, they do not yet express the CD4 or CD8 co-receptor on the cell surface, and therefore are referred to as double negative (DN) thymocytes. In early DN thymocytes both TCR gene loci are still in germline configuration. In proliferating DN thymocytes, the TCR β-chain genes rearrange first. Upon successful completion, the β-chain assembles with the glycoprotein 33, an

invariant partner chain also called pTα, to form a functional heterodimeric receptor (the pre-TCR) which is then expressed on the cell surface together with the signal transducing CD3 complex (Goettrup and von Boehmer, 1993). Expression of the pre-receptor not only triggers rapid proliferation and prevents further β-chain rearrangement; it also induces rearrangement of the α-chain locus and expression of CD4 and CD8 co-receptor molecules. The α-chain rearrangement violates the principle of allelic exclusion and may proceed through many cycles, possibly resulting in the expression of more than one TCR. $CD4^+CD8^+$ double positive cells (DP) are short-lived, and only those expressing TCRs that interact with cortical epithelial MHC-peptide complexes with a low but measurable avidity/affinity will be positively selected, i.e. rescued from undergoing apoptosis. Depending on the nature of the TCR-peptide-MHC interaction, thymocytes are directed into the $CD4^+CD8^-$ (MHC class II-restricted) or into the $CD4^-CD8^+$ (MHC class I-restricted) lineage.

Negative selection mainly takes place in the thymic medulla where it is mediated by dendritic cells. It ensures that any T cell that, at the time of maturation, is triggered to respond to intrathymically presented self-antigen above a certain signaling threshold will be removed by apoptosis (clonal deletion) or silenced by functional inactivation (*anergy*; Ramsdell et al., 1989). An estimated one third of positively selected T cells are deleted during negative selection by bone-marrow-derived APC (van Meerwijk et al., 1997). Recent findings describe the expression of tissue-specific antigens in the thymus – a phenomenon termed *ectopic* or *promiscuous* gene expression, which appears to be a specific feature of the thymus (Hanahan, 1998). The cellular and molecular regulation of such 'promiscuous' intrathymic expression is still poorly understood and has only recently been addressed by Klein et al. (2000) and Derbinski et al. (in press) who could show the intrathymic expression of several antigens is localized to medullary epithelial cells. The mechanism how self-tolerance induction through expression of tissue antigens in mTEC operates has yet to be unraveled. One possibility may be that it does not occur by T cell deletion but by generation and selection of regulatory T cells (T_{reg}). It could be shown that the generation of antigen-specific $CD4^+CD25^+$ T_{reg} cells appeared to be dependent on the expression of a neo-self antigen in radio-resistant T cells of the thymus (Jordan et al., 2001).

Furthermore, MHC molecules may also shape the T cell repertoire postthymically, as these molecules seem necessary for the survival and homeostasis of naive cells in the peripheral lymphoid organs (Goldrath and Bevan, 1999).

2.3.1 Peripheral Tolerance Induction

Clonal deletion in the thymus is quite an efficient process, and results in a highly selected T cell repertoire. However, as some self-antigens are not permanently available or expressed in primary lymphoid organs but are expressed exclusively in certain tissues or at certain developmental stages, this process alone cannot be sufficient for full tolerance induction.

While immature T cells are programmed to die when stimulated with high affinity/avidity, mature, peripheral T lymphocytes are 'designed' to respond by activation upon encountering their matching antigen. Once activated, reactive T lymphocytes may proliferate and be eliminated by a process referred to as *clonal exhaustion*. Such exhaustion of an immune response leads to the absence of immunological memory and is thought to result from permanent exposure to high antigen doses (Moskophides et al., 1993).

Peripheral tolerance induction does not necessarily go along with physical elimination of T cells. *Clonal anergy* describes the 'silencing' of a certain auto-reactive subset of T lymphocytes, i.e. it is no longer able to respond to antigenic stimulation (Nossal et al., 1994). These cells did not receive the second signal necessary for proliferation and differentiation. In some cases, a state of tolerance is reached by down-regulation of the TCR and co-stimulatory molecules (Rocha and von Boehmer, 1991; Schönrich et al., 1991; Ferber et al., 1994).

Other mechanisms of peripheral tolerance induction consist of simple anatomic impediments: Homing receptors restricting T cells to secondary lymphoid tissues exclude recognition of most antigens expressed in non-lymphoid tissues. The expression of MHC class II molecules is restricted to antigen presenting cells, (Hämmerling and McDevitt, 1974; Hämmerling et al., 1975) and is not found on non-lymphoid cells, thereby preventing activation of those $CD4^+$ T cells gaining access to non-lymphoid areas. In immune-privileged regions such as brain, additional protection is provided by the blood-brain barrier that prevents lymphocytes from entering the central nervous system. In other organs such as gonads, placenta and eye, foreign antigen cannot initiate immune responses. This is partly due to their expression of immunosuppressive cytokines and cell surface ligands, e.g. the CD95-ligand (Fas-ligand), which results in killing of previously activated T cells that normally express Fas (Bellgrau et al., 1995; Mondino et al., 1996). The situation whereby potentially self-reactive T cells are separated from their cognate antigens by anatomic or functional (e.g. missing homing receptors) barriers is called *clonal ignorance*.

After leaving the thymus, T cells re-circulate between the blood, lymph and secondary lymphoid organs. For activation and induction of immunity, certain requirements have to be

fulfilled. Naive cells, i.e. all those never having encountered their cognate antigen in the periphery, leave the blood and enter lymph nodes using specialized molecules like selectins which are lost after activation and replaced by homing receptors for non-lymphoid tissues. In the T cell areas of lymph nodes or the marginal zone of the spleen, T lymphocytes may encounter antigen that has been either swept in passively from the surrounding tissue or presented by immigrated APC. The peripheral T cell pool is obviously tightly regulated in size (Rocha et al., 1995). This may be a result of competition for limiting niches such as availability of interdigitating DC in the T cell zones, or growth factors and cytokines necessary for proliferation and/or survival. In secondary lymphoid organs potentially auto-aggressive T cells appear to be under control of a specialized lymphocyte population, the so-called *regulatory T cell subset*. This mechanism is also referred to as *active* or *dominant* tolerance and can be transferred from one animal to another (Quin et al., 1993; Saoudi et al., 1996). Furthermore, experimentally induced immune reactions could be suppressed by transfer of antigen-specific Th2 clones. In addition to the 'classical' pattern of interleukins, these regulatory T helper subsets secrete the *transforming growth factor-β* (TGF-β), an anti-inflammatory and cell-growth inhibiting cytokine (Chen et al., 1994). Shifts from a Th1 type response to a Th2 have been termed *immune deviation* (Röcken and Shevach, 1996).

Apart from these features, activation by microbial antigens is dependent on inflammation at the site of entry, that is the production of inflammatory mediators and cytokines by macrophages and parenchymal cells. Peripheral tolerance to regulated self-antigens (antigens that appear after a mature T cell repertoire has been formed) seems to be induced as an abortive form of T cell activation, which is a consequence of the absence of an inflammatory environment (Mondino et al., 1996).

Another possibility to tolerize T cells in the periphery has been under recent discussion and involves a subset of $CD4^+$ T cells, namely T regulatory cells. To date it is not clear whether $CD4^+$ regulatory T cells are a homogeneous population able to perform several different mechanisms of suppression or whether it can be divided into subsets, each specialized and phenotypically as well as functionally distinct. A population of T_{reg} was described that is constitutively present as a non-proliferative $CD4^+CD25^+$ subpopulation in lymph nodes and spleen of mice (Thornton et al., 1998), and that is able to suppress T cell responses independent of cytokines in a direct T cell contact-dependent fashion. On the other hand the existence of a $CD4^+CD25^+CD45RB^{low}$ T cell population was reported that could be expanded *in vitro* with IL-10 and secrets immunosuppressive cytokines such as IL-10 and/or TGF-β (Groux et al., 1997).

3 The Acute Phase and C-reactive Protein

3.1 The Acute Phase

The acute phase response refers to the global response of the body to the invasion of foreign agents after injury, infection or trauma. The aim is to prevent any further damage, deplete the infectious organisms and initiate wound healing. The acute phase includes drastic changes in the metabolic and biosynthetic profiles of many organs resulting in fever, the mobilization of neutrophils, an increased activation of B- and T lymphocytes as well as major shifts in the types of serum proteins synthesized by hepatocytes, especially of the so-called *acute-phase proteins*. All these effects result from the action of IL-1, IL-6 and TNF-α, cytokines released by activated macrophages (Baumann and Gauldie, 1994).

3.2 Acute Phase Proteins

Acute phase proteins (APP) such as the C-reactive protein (CRP) and serum amyloid P component (SAP) act as activators of the complement cascade and also as opsonins (Janeway and Travers, 2001). Unlike minor acute-phase proteins with basal levels that are elevated 2-5-fold upon induction, the concentration of major acute-phase proteins may be increased up to 1000-fold within a few hours (Steel and Whitehead, 1994).

Hepatocytes are the main source of CRP production. Upon stimulation by bacterial lipopolysaccharide (LPS), monocytes and macrophages start secreting IL-1 and IL-6. These cytokines in turn induce CRP synthesis and secretion by hepatocytes (Mazlam and Hodgson, 1994). 12 to 24 hours after the initial stimulus, CRP serum concentrations reach their maximum, thereafter declining to their basal levels by 72 to 96 hours. Usually only one APP functions as the major acute-phase reactant in a given species: In humans CRP is the major acute phase protein with a basal level of ≤1 μg/ml and a wide induction range (up to 1000-fold), whereas in mice, only slight increases of mouse CRP (mCRP) can be observed during acute-phases. In mice, SAP is the inducible component while in humans SAP levels of 30 μg/ml are expressed constitutively (Pepys et al., 1979; Steel and Whitehead, 1994). Within a species, the constitutive serum concentrations of some acute-phase proteins may vary significantly. In inbred mouse strains, the concentration of SAP in the absence of inflammatory stimuli differs very much and is genetically determined. Thus, the basal level in C57BL/6 mice is relatively low (5-10 μg/ml).

CRP as well as SAP belongs to the pentraxin family of proteins. They are homologous in structure and in their function and bind to the phosphoryl choline (PC) portion of cell wall polysaccharides of certain bacteria and fungi as well as necrotic or apoptotic cells. It is involved in activating the classical complement cascade, which in turn induces direct lysis of the microorganisms. (Pepys and Baltz, 1983; Steel and Whitehead, 1994). Acute-phase proteins are highly conserved during evolution and appeared earlier than the immunoglobulins. Proteins with structural and functional homologies having been found in many mammals, fishes, and even in a species of crab (*Limulus polyphemus*, Sarikaputi et al., 1991). High evolutionary conservation and the fact that to date no reports of individuals lacking hCRP have been described hint at an essential function of this protein. Apart from its ability to bind to the ubiquitously present PC, it also binds to the nuclear components chromatin, histones, small nuclear ribonucleoprotein (DuClos et al., 1988; DuClos, 1989; Volanakis et al., 1990) and to the extracellular matrix proteins fibronectin and laminin (Tseng et al., 1989). Only recently it has been shown that CRP binds to FcγR (Stein et al., 2000).
By binding material derived from necrotic cells, hCRP may prevent autoimmunity to nuclear antigens (Steel and Whitehead, 1994). In addition, a role in tumor defense by the activation of macrophages and monocytes has been discussed (Barna et al., 1994).

4 Aim of the Thesis

Understanding the requirements for T cell activation is one of the fundamental issues in basic immunology as well as regards the design of immune-based therapies.
Successful induction of an adaptive immune response depends on an appropriate antigen dose, duration of antigen presentation and the cellular environment in which antigen is presented. These parameters may vary according to the particular epitope recognized by T- or B cells. The *in situ* analysis of clonal or polyclonal T cell populations during the immune response has only recently become amenable to precise experimental analysis. Clusters of proliferating T lymphocytes have been shown to localize in proximity to dendritic cells (Ingulli et al., 1997), which are the MHC class II-expressing APC detectable in T cell areas of lymph nodes. Immigrated T cells interact with these DC and thus appear clustered. Several *in vitro* and *in vivo* studies initiated an ongoing debate about the duration, localization, dynamics and frequency of T cell/DC interactions required for full T cell activation.
Here, a new approach is introduced to address the question of early T cell activation with respect to localization and time. We have been able to isolate T cell/APC clusters directly *ex*

vivo from non-immunized and immunized animals by adopting and modifying a method previously described by Kyewski et al. (1986). We have defined these clusters as functional *in situ* units and compared the time and localization of T cell activation in different experimental models including both, $CD4^+$ and $CD8^+$ T cell as well as different MHC backgrounds. This approach for the first time provides a 'bridge' between existing *in vivo* and *in vitro* experiments.

Materials and Methods

Materials

1 Chemicals

If not stated otherwise, the chemicals were obtained from Sigma, Deisenhofen.

2 Buffer and Media

2.1 Buffer and Media for Molecular Biology

If not stated otherwise, Millipore water was used and the buffer and media were autoclaved.

TE: 10 mM Tris-HCl; 1mM EDTA, pH 7.5

TAE 1x: 40 mM Tris/acetate pH 7.5, 1mM EDTA

Loading buffer 10x: 50% (v/v) glycerol (Roth GmbH, Karlsruhe); 10% (v/v) SDS (BioRad Laboratories, Hercules, USA); 0.5% (v/v) bromophenol blue; 0.5% (v/v) xylene cyanol; 0.1 M EDTA (Fluka Chemie AG, Buchs, Switzerland)

2.2 Buffer and Media for Cell Biology

RPMI 1640 or Iscove's (media and additives were obtained from Life Technologies, Eggenstein), supplemented with 5-10% (v/v) FCS (BiochromKG, Karlsruhe), HEPES 10 mM (pH 7.3), L-glutamine 2 mM, β-mercaptoethanol 50 μM, streptomycin 50 μg/ml and penicillin 50 U/ml. Fetal calf serum (FCS) was heat-inactivated at 56°C for one hour.

Freezing buffer: 40% RPMI; 20% DMSO (dimethylsulfoxide); 40% FCS

PBS (phosphate buffered saline) 10x, pH 7.2-7.4: 8 g/l NaCl; 200 mg/l KCl; 1.470 g/l $Na_2HPO_4x2H_2O$; 200 mg/l KH_2PO_4

3 Oligonucleotides (Primers)

All primers were synthesized in the central division of the DKFZ (laboratory Dr. W. Weinig). The following oligonucleotides (sequences were obtained from c-DNA) were used for the genomic amplification of the β-chain of the Sep-tg T cell receptor by PCR:

β-chain rearrangement:

Vβ8.3 officially named BV8S3 (Arden et al., 1995), Dβ (Siu et al., 1984) and Jβ1.6 (Gascoigne et al., 1984):

5'primer: TCK 3 5'-GCA-TAC-TCG-AGT-CGC-GAG-ATG-GGC-TCC-3'
3'primer: LK 4 5'-CCA-AGA-CCG-CGG-TCA-TCC-AAC-ACA-G-3'

For PCR amplification of the variable regions of the Dep-tg T cell receptor α-chain the following primers were used:

5'primer: LK1 5'-GAG-GAT-CCC-GGG-GAT-TGG-ACA-GGG-GCC-3'
3'primer: LK2 5'-CAG-GCG-GCC-GCA-TTG-TTC-TTC-CAA-AAT-AC-3'

4 Proteins and Peptides

4.1 hCRP

Human CRP was obtained from Sigma, Deisenhofen or one of our collaborators, Prof. Dr. MB Pepys, Royal Hospital, London, UK. The protein was purified from sera of patients showing inflammatory reactions. Highly purified hCRP (99.5%) was used for immunizing mice whereas proliferation experiments and restimulation experiments were carried out with hCRP of 95% purity.

4.2 OVA

Chicken ovalbumin grade 5-6 was obtained from Sigma and diluted from a stock solution concentrated at 10 mg/ml DMSO.

4.3 Peptides

Peptides were synthesized in the core facility for peptide synthesis at the DKFZ. The peptides include the subdominant epitope region A (peptide 81-92) and dominant epitope region B (peptide 89-100) of hCRP. The core regions of the peptides do not overlap. Furthermore, no reactivity for the gap region could be found (Döffinger et al., 1997). The peptides were dissolved in DMSO at a concentration of 10 mg/ml (stock solution).

For stimulation of OVA-specific T cells either complete OVA or OVA-peptide 257-264 (SIINFEKL) or the weak agonist SIINFEDL (variant D7, Jameson et al., 1993) were used. The peptides were dissolved in DMSO at a concentration of 10 mg/ml (stock solution).

5 Antibodies

Antigen	Clone	Species/Isotype	Conjugate	Reference/Source
BrdU	3D4	mouse	PE	BD Pharmingen
CD3ε	500A3	hamster, IgG	biotin	Havran et al., 1987
CD4	H129.9	rat, IgG2b	biotin, PE, Red 613	Gibco
CD4	H129.19	rat, IgG2a	FITC, CyChrome	BD Pharmingen
CD8	53-6.7	rat, IgG2a	FITC, PE, Red 613	Gibco
CD11c	HL3	hamster, IgG	PE	BD Pharmingen
CD25	PC61	rat, IgG1	PE	BD Pharmingen
CD44	IM7	rat, IgG2a	PE	BD Pharmingen
CD45RB	16A	rat, IgG2a	PE	BD Pharmingen
CD62L	Mel-14	rat, IgG2a	PE	BD Pharmingen
CD69	H1.2F3	hamster IgG	PE	BD Pharmingen
TCR Vα11	RR8-1	rat, IgG2b	supernatant	Jameson et al., 1991
TCR Vβ5.1,5.2	MR 9-4	mouse, IgG1	biotin, FITC	BD Pharmingen
TCR Vβ8.3	1B3.3	hamster, IgG	FITC	BD Pharmingen

Secondary Reagents for FACScan Analysis

Biotinylated antibodies were detected with streptavidin-FITC (Boehringer), streptavidin-PE (Pharmingen), streptavidin-Red 613 (Gibco) or streptavidin-Cy-3 (self).

Magnetic beads

For magnet-activated cell sorting (MACS), anti-B220-, anti-CD8-, anti-CD11c- and streptavidin-beads (Miltenyi, Bergisch Gladbach) were used.

6 Animals

6.1 Wildtype strains

Mice of the inbred strains C57BL/6 (H-2^b) and Balb/c (H-2^d) were obtained from Charles River WIGA (Sulzfeld) and kept under specific pathogen-free (SPF) conditions in the DKFZ animal house.

6.2 TCR Transgenic Mice

6.2.1 hCRP

Transgenic mice expressing T cell receptors specific for either of two epitopes of the human C-reactive protein were generated in co-operation with Prof. U. Rüther, Hannover (Klein, 1997). The transgenic TCR specific for the subdominant epitope expresses a Vα4/β8.3 combination; the one recognizing the dominant epitope carries a Vα11/β5.1 combination. T cells expressing the transgenic receptor differentiate into CD4 positive cells, while CD8 positive cells additionally make use of an endogenous α-chain. Positive selection of transgenic $CD4^+$ lymphocytes is very efficient, with more than 80% of the peripheral population carrying the transgenic TCR with physiological density.

6.2.2 OVA

OT-I (CD8) and OT-II (CD4) TCR transgenic mice recognizing the dominant OVA epitope SIINFEKL have been described previously (Hogquist et al., 1994; Barnden et al., 1998). Both were bred on the C57BL/6 background for more than 10 generations. OT-I mice were kindly provided by Dr. Andreas Limmer, ZMBH, OT-II animals were obtained from Dr. Manfred Lutz, Erlangen.

DO11.10 mice, which recognize the dominant OVA peptide 323-339 in the context of H-2^d, (Ingulli et al., 1997) were kindly provided by Dr. Schimpl, Würzburg.

Methods

7 Molecular Biology Methods

7.1 Genotyping of Mice

7.1.1 Determination of DNA Concentrations

Material:
Quartz cuvettes, Spectrophotometer (Amersham Pharmacia Biotec, Freiburg)

To determine the concentration of DNA in a solution, the sample was diluted appropriately, and the photometric absorbances at 260 and 280 nm were then measured relative to a distilled water blank. The concentration of dsDNA (μg/μl) can be calculated using the standard formula

$$\mathbf{c = A_{260} \times 0.05 \times N}$$

with 0.05 being the standard absorption coefficient for DNA and N the dilution factor.
i.e. A_{260} of 1.0 results from a dsDNA solution of concentration 50 μg/μl
Purity of DNA can be estimated by dividing A_{260} by A_{280}. Values of 1.7-1.8 for DNA indicate good purity; lower ratios indicate the presence of protein.

7.1.2 Polymerase Chain Reaction (PCR)

Material:
10x concentrated primers (2.5 μm each); 10x conc. dNTP mixture (dATP, dCTP, dGTP, dTTP); Taq polymerase, 10x buffer (100 mM TrisHCl, pH8.3, 500 mM KCl, 11mM $MgCl_2$ (all: MBI Fermentas, Vilnius, Lithuania), mineral oil, RoboCycler®40 Temperature Cycler (Stratagene, Heidelberg)

The technique of amplifying DNA by polymerase chain reaction (PCR) was used to identify TCR transgenic offspring. The reactions were carried out in a small volume of 25 μl, mixing 250 nM of each primer, 10x buffer, 200 μM dNTP mix, 1.1 mM $MgCl_2$, 250 ng genomic DNA and 0.5 U Taq-polymerase, sealing with mineral oil. As PCR was very sensitive, contamination had to be strictly avoided by using freshly autoclaved water as well as pipette filter tips. Thirty cycles with the following parameters were carried out: denaturation of DNA (94°C, 90 s), primer annealing at optimum temperature (54°C, 90 s), and chain elongation for

2 minutes at 72°C. The initial denaturation step was extended to 150 s to ensure strand separation the last cycle was followed by an elongation phase of 3 minutes to ensure that all primers had been fully extended. 10 µl of each sample was loaded onto an agarose gel.

7.2 Gel Electrophoresis of Nucleic Acids

Material:
Agarose; ethidium bromide UltraPure™ (Gibco, BRL Life Technologies, Eggenstein); TAE-buffer; 100 bp ladder (Gibco, BRL Life Technologies, Eggenstein); UV-Screen (Kodak Banda, Wiesloch), Sony Video Graphic Printer UP 860 CE

PCR products (see MM 7.1.2) were checked in agarose gels (1.2% in TAE, stained with ethidium bromide in a final concentration of 0.03 µl/ml gel). Size fractionation was carried out in TAE-buffer at 70 V. DNA fragments could be visualized and documented by photography using UV light (254 nm).

8 Immunological and Cell Biology Methods

8.1 Cell culture

8.1.1 Cultivation of Eukaryotic Cell Lines

Passaging of cell occurs under sterile conditions in a laminar flow (The Baker Company Inc. Stanford, USA). Cells and cell lines were cultured in complete RPMI 1640 or Iscove's (see MM 2.2) at 37°C, 7.5% CO_2 and 96% humidity (Hereaus incubator).

8.1.2 Freezing and Thawing of Cells

Material:
Freezing buffer; RPMI 1640, complete with 5% FCS; 2 ml cryovial tubes (Nalgene Cryoware™ Rotheras, Hereford, UK); Minifuge T (Heraeus, Hanau)

Cells were prepared by resuspending them in freezing buffer ($1x10^6$-$5x10^6$ cells/500 µl) and immediately placing them at -70°C. DMSO diffuses into the cells, thus preventing the formation of crystals when freezing and therefore the destruction of the cell membrane. After 1-2 days the cells were transferred to liquid nitrogen for permanent storage.

When thawing cells, it was very important that the thawing time be minimized. Frozen vials were transferred to a water bath (37°C) and, when thawed, the cells were taken up in 10 ml of fresh RPMI 1640. After centrifuging for 5 minutes at 1200 rpm, the pellet was resuspended in medium.

8.1.3 Determination of Cell Number and Viability

Material:
Trypan blue solution (0.16% (w/v) trypan blue (Boehringer Ingelheim Bioproducts, Heidelberg), 0.90% (w/v) NaCl, 0.1% NaN_3, filtered through 0.45 μm, stored at room temperature), Neubauer hemacytometer

Staining cell populations with trypan blue provides a possibility of visually distinguishing between unstained viable and blue-stained dead cells. For cell counting, a sample is mixed with trypan blue staining solution at the desired dilution factor (usually 1:5) and viable cells were counted in 4 large corner squares of a Neubauer chamber. The cellular concentration was calculated using the following formula:

$$\text{cells/ml} = \text{average cell count per square} \times \text{dilution factor} \times 10^4$$

8.1.4 Preparation of Mouse Single Cell Suspensions from Organs

Material:
Centrifuge, Minifuge T (Heraeus), 30 or 60 μm nylon gauze (Eckert, Waldkirch)

If not stated otherwise, methods were performed (with some modifications) according to *Selected Methods in Cellular Immunology* (Mishell and Shiigi, 1980).
After removal of the organs they were kept on ice in Petri dishes containing PBS or Iscove's (5-10% FCS). The spleen was gently teased with a forceps, preferably without breaking it into small fragments. Cell suspensions of lymph nodes were obtained by pressing the organs between the previously sterilized ends of two microscope glass slides, thymi were carefully fragmented with forceps and transferred into suspension by repeated resuspension with pipettes. The cells were passed through 60 μm nylon gauze to remove aggregates and debris, and were then washed twice with PBS (1200 rpm, 5 minutes). Suspensions needed for functional assays were to be handled under sterile conditions.

The nucleated cell yield per normal spleen was 0.5-1x10^8, for mesenteric lymph nodes 2-3x10^7 cells were obtained, the thymus of mice 6-16 weeks of age contained approximately 1x10^8 cells.

8.1.5 Purification of Peripheral Blood Lymphocytes

Material:
Heparin (Braun, 5000 I.U./ml), Ficoll Histopaque-1077

Peripheral blood was used to detect populations of transferred TCR transgenic lymphocyte populations in hCRP transgenic or C57BL/6 mice. After warming the animals using red light, 4-5 drops of blood were extracted from the tail vein, preventing it from clotting by adding heparin-buffer (200 µl PBS, 50 U heparin/ml blood, FCS 2%). Leukocytes were purified on a Ficoll gradient at 1200 rpm for 20 minutes (RT, no brake). Cells recovered from the interphase were washed twice with PBS before staining. Purified lymphocytes could now be treated as previously described (MM 2.7).

8.1.6 Red Blood Cell Lysis

Material:
Lysis buffer (155 mM NH_4Cl; 10 mM $KHCO_3$; 100 mM EDTA)

The cell suspension to be depleted of erythrocytes was pelleted and 1 ml of lysis buffer was added to the pellet. After 90 seconds cells were washed twice in 50 ml medium or PBS.

8.1.7 Isolation of T cell/APC Clusters (Rosettes)

Material:
Collagenase/Dispase buffer [Dispase grade I 0,2 mg/ml (Boehringer Mannheim/Roche Diagnostic), collagenase grade IV 0,2 mg/ml (Interchem, München), DNaseI (50 µg/ml; ICN), Aprotinin (12 TIU/mg), RPMI 1640 supplemented with 2% FCS and 20 mM Hepes]

Preparation of lymph node clusters was performed with modifications as described by Kyewski et al., 1986. In short, pooled lymph nodes were depleted of free cells by stirring gently for 3-5 minutes at RT. Fragments were collected and digested 3-5 times (10 minutes, 27-37°C) with collagenase/dispase buffer. Clusters were enriched by sedimentation at unit gravity through FCS (2 times 10-45 minutes, 4°C).

8.1.8 Separation of Lymphocytes by MACS (Depletion)

Material:
Mini MACS (magnetic cell separators), MACS separation columns (both Miltenyi Biotec GmbH, Bergisch Gladbach), MACS buffer (PBS supplemented with or without 5 mM EDTA and 0.5% BSA),

$CD4^+$ cells were enriched by Magnetic Activated Cell Sorting (MACS). Lymph node and spleen cell suspensions were obtained as previously described (see MM 8.1.4). Cells were washed, pelleted and 10^6 to 10^7 cells were incubated with a cocktail of anti-B220 Microbeads, anti-CD8-, anti-Mac-1-, and anti-P7/7 supernatant (20 µl each, 15 min, on ice). After washing, the pellet was resuspended in 80 µl buffer, 20 µl MACS goat-anti-rat IgG Microbeads were added (15 min, on ice). Separation was performed as described (magnetic cell separation with MiniMACS, Manual, Miltenyi). In short, washed and resuspended cells were passed over the column. The effluent was collected as positive fraction, after removal from the separator magnetically labeled cells were flushed out of the column (negative fraction). To determine the enrichment, a suspension sample taken prior to the separation, as well as aliquots of the positive and the negative fraction were stained for the respective surface antigens and analyzed by flow cytometry (see MM 8.3.1).
T cell/DC clusters were enriched by labeling with azide-free antibody and sav-microbeads and subsequent passing over a column. In contrast to single-cell suspensions, the flow-through rate had to be slowed down with the help of a three-way stopcock. To avoid dissociation of the clusters, the whole procedure had to be performed with extreme care and all equipment was kept chilled. Purity was analyzed by fluorescence microscopy.

8.1.9 *In vitro* Proliferation Assays

Material:
96-microwell-plates, round-bottom (Costar, Cambridge, MA), ^{3}H-methyl-thymidine (Amersham International, Little Chalfont, UK), HL-1 medium (BioWhittaker, Wakersville, Maryland, USA) Micro Cell Harvester (Scatron, Norway), glass fiber filters (LKB-Wallac, Turku, Finland), Betaplate Harvester 1205 (LKB-Wallac)

Freshly prepared mesenteric lymph node cells were stimulated with titrated amounts of peptide at a concentration of 5-6x10^4 cells/well in 200 µl HL-medium, supplemented as previously described (MM 2.2), for 60 hours and 20 µCi/well ^{3}H-methyl-thymidine (Amersham) was added for the last 12 hours. Cultures were performed as triplicates. They were harvested on glass fiber filters and the incorporation of radioactive thymidine was measured by liquid scintillation counting using a Betaplate Harvester (Wallac, Freiburg).

8.2 Immunochemistry

8.2.1 Preparation of Antibody Supernatants

Material:
24-well plates (Costar), 100 ml and 500 ml culture flasks (Falcon), 1500 ml spinner flasks, Hereaus incubator (Heraeus, Hanau); Sorvall-centrifuge H6000A

Antibody secreting hybridomas were incubated with RPMI 1640 (complete, 5% FCS) until grown to an approximate density of 10^6 cells/ml. As the average doubling time of hybridomas is normally between 12 and 24 hours, they were sub-cultured and expanded every 2-4 days by splitting the suspension and transferring some of it to a larger culture flask and medium was replenished. Depending on growth, cells were transferred to spinner flasks containing 500 ml of RPMI 1640 (complete, 5% FCS) and were incubated at 37°C for another 2-3 weeks. Medium was added at regular intervals to yield a final volume of 1.5 liters/flask. The cells were depleted (Sorvall, 10 minutes at 4°C, 3000 rpm) and sodium azide was added to the supernatant in final concentration of 0.1%. The antibody could now be purified or supernatant was frozen at - 20°C.

8.2.2 Purification of Antibodies

Material:
RC-5 Superspeed Refrigerated Centrifuge (Sorvall); Falcon tubes (Falcon); sterile filters, 5 μm, pyrogen free (Schleicher & Schuell, Dassel); 0.22 μm sterile filters (Millipore, Bedford, USA); protein G Sepharose column (Pharmacia, Freiburg); Amicon chamber (Minicon-B, Amicon, Danvers, USA)

Purification of the antibody solution was performed by affinity chromatography over a protein G bead column. The enriched antibody preparation was passed through a protein G bead column. The column was washed twice with 200-300 ml citric acid pH 5.0 to remove impurities, was eluted with citric acid pH 2.8 and 1 ml fractions were collected in Eppendorf tubes containing 500 μl of 1 M Tris pH 8.0 to bring the pH back to neutral. The antibody-containing fractions were identified by absorbance at 280 nm and positive fractions [OD_{280} higher than 0.14 (0.1 mg/ml)] were pooled and then dialyzed against PBS containing 0.1% sodium azide for 1-2 days. Again, the protein concentration was measured and either sodium azide (0.1%) was added or the solution was sterily filtrated (mesh 0.22 μm). Finally, the antibody was concentrated to a concentration of ≥ 1 mg/ml (Amicon chamber).

8.2.3 Biotinylation of Antibodies

Material:
Biotin-X-NHS (Calbiochem Bad Soden); dimethylformamide (freshly prepared, 1 mg/ml)

Purified antibodies incubated with biotin-X-NHS (100 µg biotin-X-NHS dissolved in dimethylformamide/mg antibody) were rolled o/n at 4°C. Non-bound biotin was removed by dialysis against PBS.

8.3 Immunofluorescence Analysis

8.3.1 Analysis of Cells Using Flow Cytometry

Material:
TCR transgenic cells, polystyrol tubes (Greiner, Nürtingen), FACS buffer; PBS, 5% FCS, 0.1% (w/v) NaN_3, FACScan (Beckton-Dickinson, Heidelberg)

Expression of cell surface antigens was detected by FACS analysis. For staining, 1-5x10^6 cells were incubated with the according antibodies for 10-15 minutes at 4°C. They were washed once in FACS-buffer and then resuspended in 150 µl FACS-buffer. For flow cytometric analysis, data of 10,000 stained cells were collected and analyzed with a FACScan and Cellquest research software (Becton Dickinson, Apple Macintosh). Non-lymphoid and dead cells as well as cell fractions could be excluded from data collection by appropriate forward- (cell size) and side scatter (granulation) gating.

8.3.2 Intracellular Staining of T cells with the Viable Dye CFSE

Material
PBS/0.1% BSA, 5-, 6-diacetate-*c*arboxy*f*luorescein*s*uccinimidyl*e*ster (CFSE, Molecular Probes, Oregon, USA)

The cells to be intracellularly stained with CFSE were washed twice in PBS, and were then resuspended at 1-5x10^7 cells/ml. CFSE was added to a final concentration of 2-5 µM, and the suspension was incubated at 37°C for 10 minutes. The reaction was stopped by adding cold PBS/10%FCS. To remove dead cells and debris, cells were filtered through 30 µm nylon mesh (Nybolt, Waldkirch). A cell loss of up to 30% had to be expected.

8.3.3 Intracellular Staining of T cells with the Viable Dye CMTMR

Material
PBS, CellTracker™Orange (CMTMR, Molecular Probes, Oregon, USA)

The cells to be stained with CMTMR were washed twice in PBS, and were then resuspended at 1-5x10^7 cells/ml. CMTMR was added to a final concentration of 1 μM (at higher concentrations the dye may become toxic), and the suspension was incubated at 37°C for 30 minutes. The reaction was stopped by adding cold PBS. The cells were incubated for another 30 minutes to allow all dye to be converted. The cells were washed twice. To remove dead cells and debris cells were filtered through 30 μm nylon mesh (Nybolt, Waldkirch). A cell loss of up to 30% had to be expected.

8.3.4 BrdU Analysis of Cell Division

Material:
BrdU (Sigma, Deisenhofen), PE-conjugated anti-BrdU (Pharmingen), PBS, DNaseI (ICN), DNase I buffer (4.2mM $MgCl_2$, 0.15 M NaCl, pH 5-7), 2% paraformaldehyde in PBS with 0.01% Tween-20

For short-term labeling, mice were injected intraperitoneally (i.p.) for two days every 12 hours with 60 μg BrdU/PBS per gram body weight.
Cell surface staining could be performed as previously described (8.3.1). BrdU staining was performed with little modification as previously described (Tough and Sprent, 1994). After washing with 2 ml PBS, the pellet was resuspended in 0.5 ml ice cold PBS and 1.2 ml 95% EtOH were added drop-wise while gently vortexing (to prevent T cell clumping). The cells were incubated on ice for 30 minutes and were then washed with 2 ml PBS. They were fixed with 1 ml 2% paraformaldehyde/PBS/0.01% Tween for 30 minutes at room temperature. Washing with 2 ml PBS was followed by incubation with 0.5 ml DNase (100 Kunitz units/ml in DNase buffer) for 10 minutes at room temperature. They were again washed with 2 ml PBS and incubated with anti-BrdU mAb for 30-60 minutes at RT. To allow non-bound antibody to leave the cells, they were washed and incubated another 10 minutes with PBS at RT.

8.3.5 Immunofluorescence Microscopy

After fixation with 2% PFA and appropriate staining, confocal microscopy was performed with a Zeiss LSM410 (later replaced by model LSM510) with the help of Dr. Herbert Spring, normal analysis was performed with a Zeiss Axiophot and Axiovision 3.0 software.

8.4 Animal Experimental Methods

8.4.1 T cell Transfers

For cell transfers, spleen and mesenteric lymph node cells of TCR transgenic mice were prepared (see MM 8.1.4) and pooled. After red blood cell lysis (see MM 8.1.6), cells were analyzed for the frequency of TCR-tg T lymphocytes by FACS analysis. 4-5x10^6 transgenic cells/mouse were resuspended in 100 µl PBS and adoptively transferred intravenously (i.v.) into C57BL/6 or Balb/c mice.
Transfers were also performed with intracellularly stained cells.

8.4.2 Immunization

Material:
Metofane (Janisen-Cilay), antigen, PBS, Complete Freund's Adjuvant (CFA, Sigma, Deisenhofen)

Mice were anaesthetized by inhalation of Metofane and immunized into the hind footpads with antigen/PBS in CFA, total volume 50 µl/foot.

Results

1 Isolation *ex vivo* and Characterization of T cell/APC Clusters

1.1 Isolation and Enrichment

The method of preparing T cell/APC clusters from thymus has been previously described (Kyewski et al., 1987). Here, this method was modified to obtain such clusters from lymph nodes. In most cases draining lymph nodes from animals previously immunized with antigen in CFA or CFA alone were prepared. To facilitate access to the T cell areas during the following collagenase/dispase digests, the capsules were carefully torn open with two syringes. The tissue fragments were transferred to small tubes and covered with RPMI containing 5% FCS. The tube was placed in a heated water bath and stirred for three minutes at 27°C. During this step non-associated ('free') cells were washed out. The fraction was collected and used as control fraction, referred to as 'free cells'. The remaining tissue was now covered with collagenase/dispase and four subsequent digests at increasing temperature were performed. Each fraction was collected and microscopically analyzed for the content of clusters. Appropriate fractions were pooled and passed through 60µm nylon gauze. Clusters were enriched by two subsequent FCS-gradients at unit gravity. This way, a purity of 1:1 to 1:10 (clusters:free cells) could be obtained. Staining of the dendritic cell marker CD11c revealed that approximately 70% of the clusters contained a DC as central APC. The average number of T cells clustered around each APC was 10-20.

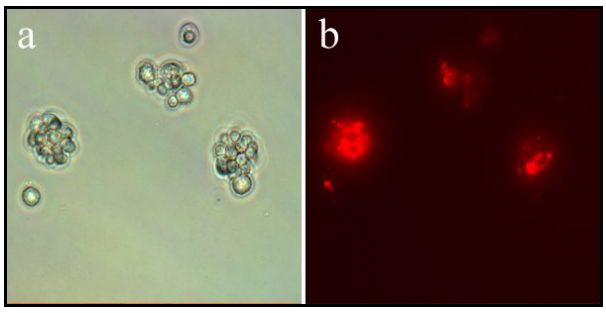

Figure 1 T cell/DC-clusters. Clusters were prepared from the draining lymph nodes of CFA immunized animals as described, and DC were stained with anti-CD11c-PE. (a) phase contrast, (b) fluorescence. Magnification: 400-fold.

1.2 Cluster Purification via MACS

To further enrich DC-containing clusters, the method of magnetic cells sorting (MACS) was adopted and modified. Compared to single cell sorting, sorting of clusters turned out to be more difficult: First, as clusters are multicellular complexes, they are much heavier than single cells and the thus 'extra' weight of the T cells surrounding the central APC has to be compensated by sufficient staining with beads. Additionally, the interactions holding clusters together are rather sensitive to substances interfering with receptor mediated interactions, such as trypsin, azide or depletion of divalent cations. Therefore the MACS-beads had to be removed from their original, azide-containing buffer and taken up in PBS. This was done by loading them onto a MiniMACS column, washing twice with PBS and flushing them out without diluting them. The purified, concentrated beads were used to label freshly prepared clusters in a volume of 50-100 µl for 30 minutes. After washing, the clusters were loaded onto a column. To minimize the flow-through rate and thus provide more time for labeled cells to bind to the column, a three-way stopcock was used. Because of the size of multicellular T cell/APC complexes we were faced with the problem of removing successfully loaded clusters from the columns by applying pressure low enough not to destroy them. Instead of using normal Mini- or Midi-MACS columns, we chose columns designed for separating large cells such as megakaryocytes (see Miltenyi, Manual). After washing twice to deplete non-bound cells, the column was removed from the magnet and the clusters flushed out by quickly pipetting PBS through the column.

Even though this method principally worked and separation of multi-cellular aggregates could be shown for the first time, it turned out to be too tricky and time consuming to apply it routinely.

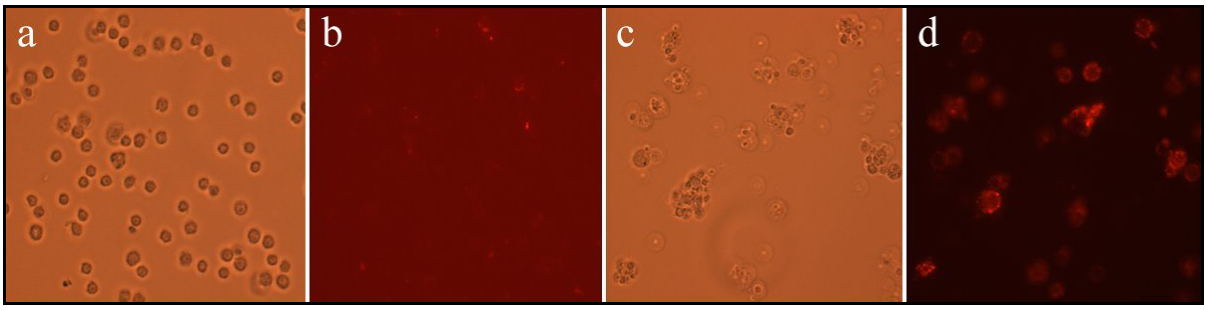

Figure 2 MACS-enriched clusters. Here, enrichment of MHC class II-positive thymic clusters, which were used to set-up this method, is shown. (a) and (b) depict the MHC class$II^{neg/lo}$ fraction of a thymic cluster preparation after MACS enrichment. In (c) and (d) the MHC class II^{high} fraction is shown. Magnification: 250-fold.

1.3 Stable Cluster Formation Is Antigen-independent

The formation of DC/T cell clusters did not appear to be strictly dependent on deliberate antigen administration as it was also possible to isolate them in low numbers from non-activated lymph nodes (even though here we cannot exclude an ongoing host infections). Nevertheless it has to be pointed out that the yield of clusters obtained from immunized animals was higher and increased with time after immunization. Furthermore, in case of antigen application, the number of clusters containing high numbers of Ag-specific T cells also increased. While from the mesenteric lymph nodes of non-immunized animals an average number of $1.5x10^4 \pm 0.2$ (n = 3) clusters could be obtained, it increased 2-10fold when preparing T cell/APC aggregates from the draining lymph nodes (inguinal and poplietal) of mice immunized with antigen in CFA. Thus, it can be concluded that clusters also form in the absence of deliberate immunization but that their formation is strongly enhanced by the presence of an ongoing local immune response.

2 T/APC Clusters as 'Functional Units' of T cell Activation *in situ*

2.1 Cellular Composition

In order to define the cellular composition of clusters, we examined the phenotype of cluster-associated versus non-associated T cells. Table I summarizes the results.

		CD4	CD8	ratio CD4:CD8	B220	CD11c
CFA	free	23.5 ± 6.8	20.1 ± 7.0	1.2 ± 0.2:1	56.8 ± 13.3	1.4 ± 1.3
	cld*	11.5 ± 2.6	10.2 ± 5.3	1.2 ± 0.4:1	69.6 ± 9.5	3.9 ± 2.5
Dep	free	14.6 ± 2.2	10.4 ± 2.0	1.2 ± 0.3:1	n.d.*	n.d.
	cld *	16.0 ± 3.0	4.4 ± 2.3	2.5 ± 0.4:1	n.d.	n.d.
Sep	free	10.8 ± 4.4	10.7 ± 2.7	1.4 ± 0.5:1	n.d.	n.d.
	cld *	14.3 ± 2.5	3.3 ± 0.3	3.2 ± 1.2:1	n.d.	n.d.
OT-I	free	18.4 ± 4.9	17.7 ± 5.2	1.1 ± 0.3:1	69.5 ± 5.7	1.4 ± 1.1
	cld *	10.4 ± 2.4	18.7 ± 5.3	0.6 ± 0.3:1	65.7 ± 8.0	7.7 ± 6.6

Table I **Cluster composition.** All values represent the mean percentage ± SD of 3-5 experiments with 3 or more time points assessed. Note changes of the CD4:CD8 ratio in the cluster-derived fraction compared to the non-associated one (highlighted in red).
* cld = cluster-derived; n.d. = not determined

CD4/CD8 T cells

In draining lymph nodes taken from animals that had been immunized with CFA only, the CD4:CD8 ratio of free and cluster-derived cells was determined on days one to three after immunization. Among free cells, a ratio of (1.2 ± 0.2):1 indicated the presence of almost equal numbers of $CD4^+$ and $CD8^+$ T cells. Among cluster-derived cells a ratio of (1.2 ± 0.4):1 could be determined. The average percentage of non-associated lymphocytes was 23.5 ± 6.8% $CD4^+$ and 20.1 ± 7.0% $CD8^+$ T cells. In cluster-derived fractions numbers were only half as high, with $CD4^+$ cells taking up 11.5 ± 2.6% and $CD8^+$ cells resembling 10.2 ± 5.3% of the lymphocyte population. In two out of three experiments an increasing number of T cells could be observed in APC association with time while T lymphocytes in the non-associated fraction decreased simultaneously. This accounted for both, $CD4^+$ and $CD8^+$.

One model used to analyze the cellular cluster composition upon Ag/CFA immunization was initially set up in our laboratory to answer questions regarding tolerance induction towards an inducible soluble serum protein, namely the human C-reactive protein (hCRP; Klein et al., 1998). It includes two T cell receptor (TCR) transgenic (tg) mouse strains recognizing either a dominant (Dep, aa 89-100) or a subdominant (Sep, aa 81-92) epitope on the $H\text{-}2^b$ background (Klein, 1997; Klein et al., 1998). Immunizing animals with a previously determined amount of hCRP allowed us to 'localize' the ongoing immune response to a limited number of lymph nodes and thus obtain enough clusters for analysis.
When comparing the cellular composition of free cells versus cluster-derived ones in the lymph nodes of animals that had been immunized with Ag/CFA and had subsequently been transferred with antigen-specific T cells on day four after immunization, a segregation of the corresponding T cells into clusters could be observed. While the CD4:CD8 ratio among non-associated lymphocytes remained constant at (1.2 ± 0.3):1 (Dep) and (1.4 ± 0.5):1 (Sep) over a time range from 16-40 hours after transfer, an increased average ratio of (2.5 ± 0.4):1 (Dep) respectively (3.2 ± 1.2):1 (Sep) in the cluster-derived fraction could be observed. In comparison to $CD8^+$ cells associated $CD4^+$ cells are enriched by a factor of 2. Interestingly, this shift did not result from an elevation of the relative numbers of $CD4^+$ cells compared to the non-associated fraction [14.6 ± 2.2% vs. 16.0 ± 3% (Dep) and 10.8 ± 4.4% vs. 14.3 ± 2.5% (Sep)] but from a decrease in the proportion of cluster-associated $CD8^+$ T cells.

In addition to the hCRP model we examined the cellular cluster composition in the OT-I model. OT-I mice have been described previously (Hogquist et al., 1994). In short, $CD8^+$

OT-I tg T cells carry a Vα2Vβ5.1 TCR and recognize the chicken ovalbumin-derived peptide SIINFEKL (aa 257-264) in the context of $H2^b$. Upon cluster preparation from mice previously immunized with OVA and transferred with OT-I cells, the CD4:CD8 ratio among non-associated lymphocytes also remained unchanged at 1.1 ± 0.3:1 over a time range from 13 60 hours after transfer. Here, in the cluster-derived fraction an average ratio of 0.6 ± 0.3:1 could be observed. Thus, associated $CD8^+$ cells are enriched by a factor of 2 in this MHC class I model. With 18.7 ± 5.3% the relative number of $CD8^+$ cells in clusters is not elevated compared to the non-associated fraction (17.7 ± 5.2%) whereas the relative number of $CD4^+$ cells in clusters is only half as high as in the non-associated population (18.4 ± 4.9 vs. 10.4 ± 2.4).

Thus, in both models the 'relative preponderance' of lineage-specific T cells in the cluster-derived fraction versus the non-associated fraction is not due to a relative increase in number of lineage-specific cells themselves but to a reduction of lymphocytes of the opposite lineage.

B cells

Several groups have reported the existence of B cell/DC clustering (Tew et al., 1990; Dubois et al., 1997; Björck et al., 1997; de Vinuessa et al., 1999). This association does involve both interdigitating DC (IDC) and follicular DC (FDC). Dubois et al. (1997) could show clustering of CD40L-transfected B cells with IDC *in vitro,* while Björck et al. (1997) found human tonsillar IDC of the T cell area to co-localize with naive B cells *in situ*. On the other hand FDC exhibit DC morphology, but are restricted to the light zone of germinal centers and lack phagocytotic activity. Instead they trap and retain immune complexes on their cell surface for prolonged periods of time. Furthermore, they have been subjected to play a role in plasmablast survival.

With an average of 56.8 ± 13.3% of non-associated and 69.6 ± 9.5% of cluster-derived cells, B cells made up a surprisingly large proportion of cells in CFA immunized animals.

In the OT-I model, the proportion of $B220^+$ lymphocytes was similar to that of CFA immunizations: with 69.5 ± 5.7% among free cells and 65.7 ± 8.0% among cluster-derived cells, the number of B cells in clusters and non-associated cells were equally high. As with our method we were not able to distinguish between clusters derived from T cell areas and follicles, it remains to be shown whether the B cells originate from follicles or T cell areas.

Dendritic cells

In mice immunized with CFA alone, the number of dendritic cells - determined by CD11c-staining of non-associated and associated cell fractions - were found to make up a small proportion. Expectedly, the numbers of DC in the non-associated fraction were lower (1.4 ± 1.3%) than those in the cluster fraction (3.9 ± 2.5%). Similar data was obtained for the OT-I model: The fraction of non-associated cells contained 1.4 ± 1.1% DC and the fraction of cluster-derived 7.7 ± 6.6%. The numbers of dissociated cells agree well with the relative frequency of DC in intact clusters (approximately 10-20 lymphocytes per cluster). It should be noted that the high SD values result from the observation that at early time points the number of DC-positive clusters was high but rapidly decreased after onset of proliferation: In one experiment numbers steadily decreased from 18.3% at 13 hours after transfer to 3.5% at 60 hours after transfer – a finding that coincides with other groups who showed that the number of DC rapidly declines after activation of lymphocytes (Ingulli et al., 1997).

2.2 Segregation of Ag-specific T cells into Clusters

As immunization resulted in a difference in the T cell composition of cluster-derived fractions versus non-associated ones, it was of interest whether Ag-specific T cells preferentially interacted with APC and whether their distribution was random or selective. To do so, mice were immunized with human C-reactive protein/CFA and four days later $5x10^6$ antigen-specific T cells were adoptively transferred, which had previously been labeled with the intracellular viable dye CFSE. At several time points after transfer, 24-48 hours, clusters were prepared as described and 100 $CD11c^+$ clusters were assessed for the number of labeled T cells they contained. Figure 3a depicts one out of three experiments performed with T cells recognizing the subdominant epitope of hCRP. It could be observed that the proportion of clusters containing one or more CFSE-labeled T cells increased over time while the number of clusters containing no labeled lymphocytes decreased at the same time. At 36 hours after transfer, the proportion of clusters containing green cells was the highest and decreased thereafter. Obtaining clusters from mice transferred with T cells specific for the dominant epitope (Dep) revealed that here the proportion of clusters containing green cells was the highest at 30 hours after transfer and decreased thereafter (Figure 3b). In both cases, a large proportion of clusters did not contain any CFSE-labeled cells at all time points. The coexistence of clusters devoid of any transferred cells and clusters with more than three

antigen-specific cells accounts for a segregation of Ag-specific T cells into clusters. Considering an average number of 10-20 T cells per cluster, 3 or more CFSE-stained T cells/cluster equals ≥20%. In comparison, only 5% of cells in the non-associated fraction are CFSE-positive at this time point. Hence, there is a 5-fold segregation of antigen-specific lymphocytes in individual T cell/APC clusters.

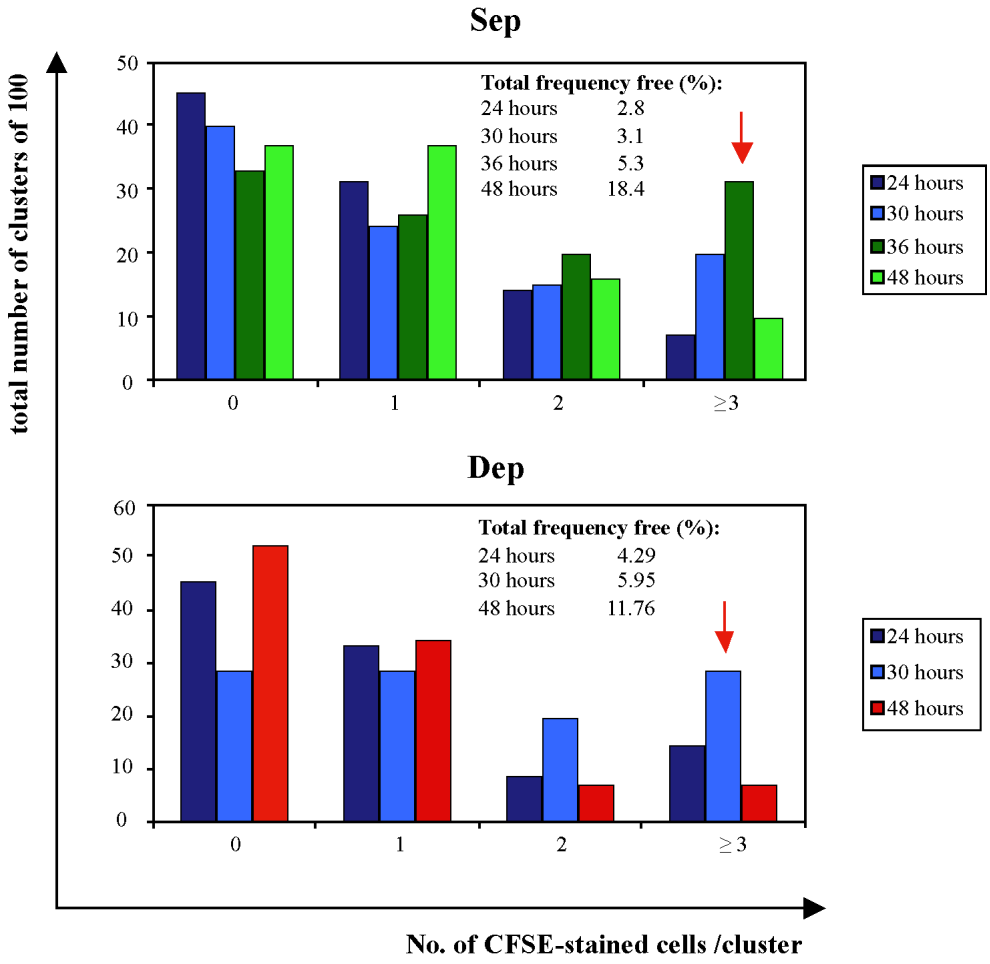

Figure 3 Segregation of CFSE-stained T cells into individual APC clusters during proliferation. Mice were immunized with 50 µg hCRP/CFA and subsequently received $5x10^6$ CFSE-labeled Sep- or Dep-specific T cells. Clusters were fixed with 2% paraformaldehyde and analyzed by fluorescence microscopy. 100 clusters were scanned randomly and the number of CFSE-stained T cells/cluster was counted. For Sep one of three experiments is shown, for Dep the only one performed. Note that for Sep-specific T cells (a) the accumulation of donor cells in clusters was highest at 36 hours after transfer while for Dep-specific T cells (b) it was highest at 30 hours after transfer.

2.3 Only Recent Immigrants Segregate into Clusters

Defining the cellular composition of clusters, we could show that antigen-specific T cells segregate into clusters. This finding led us to the question of whether APC-associated and non-associated lymphocytes differed in their functional status. We first assessed expression of the homing receptor CD62L, which is down-regulated upon lymph node entry through high endothelial venules.

Clusters obtained from the draining lymph nodes of animals that had been immunized and subsequently transferred with Ag-specific lymphocytes were subjected to a short EDTA treatment to obtain a single-cell suspension for FACS analysis. Antigen-specific T lymphocytes were identified by staining the transgenic TCR β-chain as well as the corresponding co-receptor. Only cells having down-regulated CD62L were found in clusters while on non-associated T cells it was expressed at high levels (Figure 4, p38). This finding was confirmed in four different transgenic models (Dep, Sep, OT-II – all CD4 – and OT-I – CD8) as well as in animals immunized with CFA only and implies that only recent immigrants interact with antigen-bearing APC.

2.4 Activation Status of Clustered T cells

In addition to the homing marker CD62L we assessed the activation status of clustered T cells by staining of the IL-2 receptor β-chain (CD25) and the early activation marker CD69, which has been described to be up-regulated early after activation (2-6 hours; von Stipdonk et al., 2001). Are T cells activated whilst in contact with their cognate APC or do they detach after receiving an appropriate stimulus and subsequently up-regulate activation markers?

CD69

Being one of the early events in T cell activation, the up-regulation of CD69 provided a means by which we could assess whether T cell activation is initiated while the lymphocyte is in contact with the APC or whether direct cell-cell contact is not necessary. Interestingly, strong CD69 up-regulation could be observed on cluster-derived, transgenic T cells only. This implicated that T cell/APC contact is a necessary parameter for activation (Figure 5a, p39).

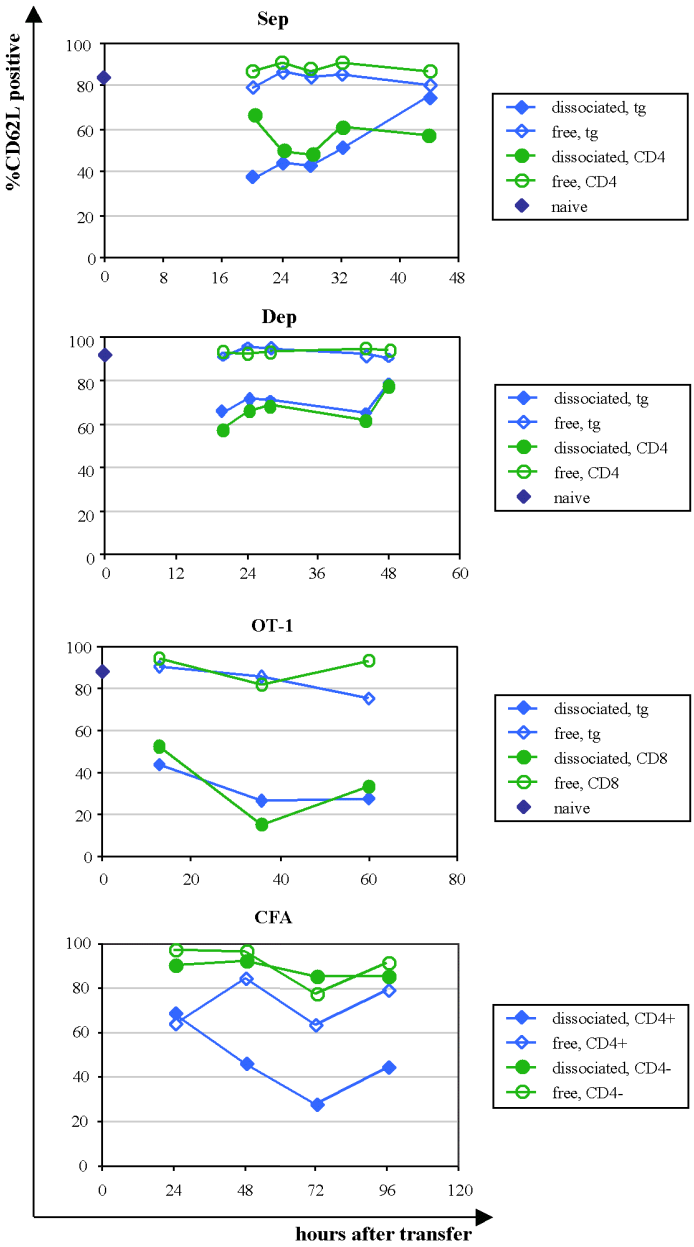

Figure 4 Segregation of CD62L^{low} T cells into clusters. Clusters were prepared at several time points after transfer. Cluster-derived and non-associated cells were analyzed by FACS for CD62L expression. Transferred cells were identified by expression of their transgenic TCR β-chain and the corresponding co-receptor. Data of 50,000 cells total were collected.

CD25

The supposition that T cell activation occurs concomitantly with APC contact was supported by the finding that in all four models investigated, CD25 was also found to be significantly up-regulated in the fraction of cluster-derived transgenic cells whereas associated non-transgenic cells showed no or only little expression (Figure 5b, p39). Furthermore, lymphocytes that were non-associated did not up-regulate this marker significantly.

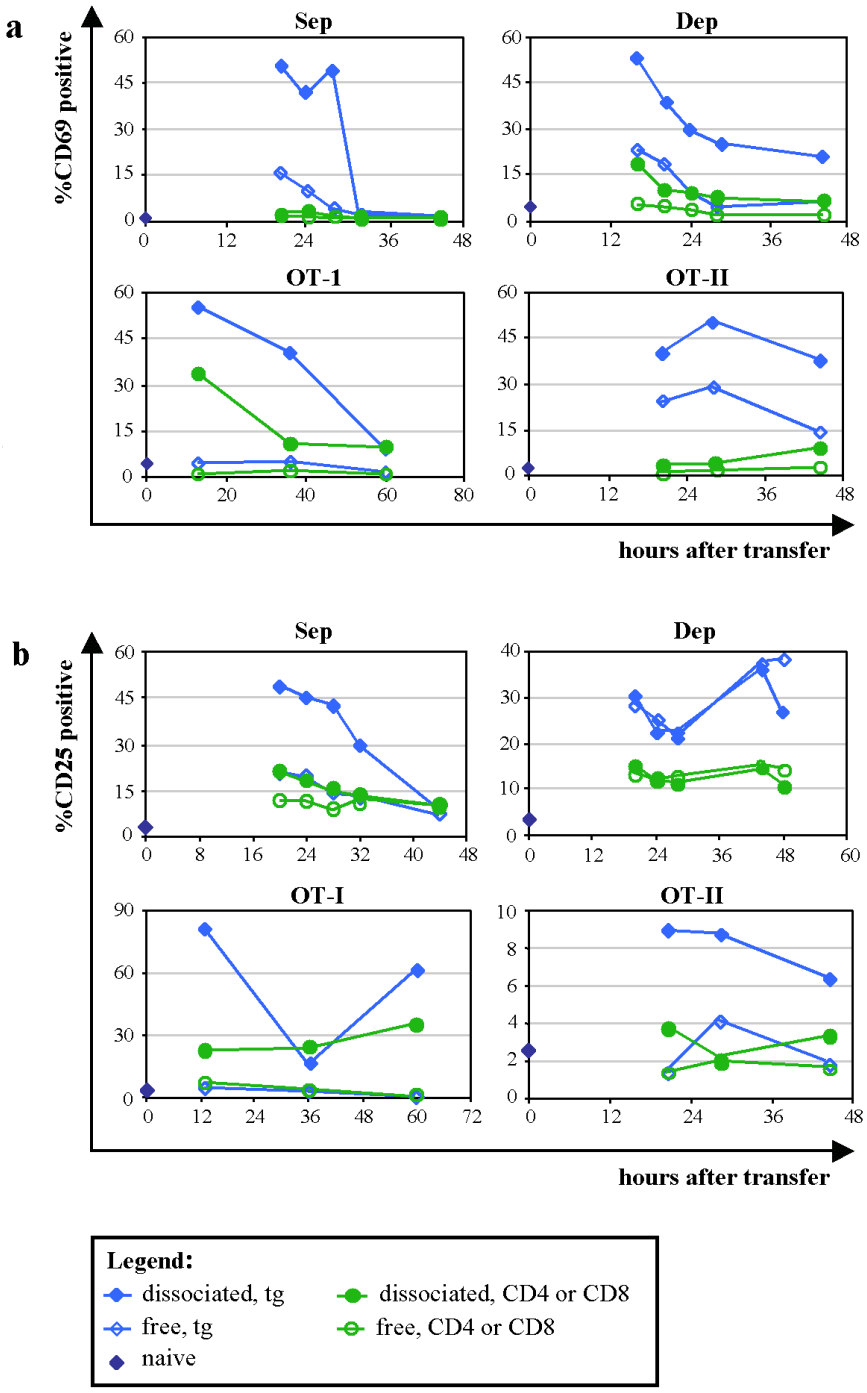

Figure 5 CD69 and CD25 are up-regulated on cluster-derived T cells only. Clusters were prepared at several time points after transfer. Cluster-derived and non-associated cells were analyzed by FACS for CD69 (a) or CD25 (b) surface expression. Transferred cells were identified by expression of their transgenic TCR β-chain and the corresponding co-receptor. Data of 50,000 cells total were collected. Note starting point of naive cells before injection.

2.5 T cell Proliferation is Initiated in T cell/DC Clusters

So far we have shown the cellular composition of clusters as well as the phenotype of both associated and non-associated T cells. The data obtained indicate that such aggregates could indeed be 'functional units of T cell activation' as has previously been proposed by several groups (Mitchison 1990; Ingulli et al., 1997; Kudo et al., 1997; Luther et al., 1997). We now wanted to address the question of whether T cells are not only activated in clusters but whether proliferation also is initiated in clusters.

To dissect the proliferation kinetics of T cells specific for a dominant and a subdominant epitope of hCRP, T cell/APC clusters were prepared at different time points and analyzed for T cell proliferation. The use of the viable intracellular fluorescent green dye CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester), which segregates equally between daughter cells during division, allowed localizing the cells of interest *ex vivo* and determining their proliferation history. Again, C57BL/6 mice were immunized in both hind footpads. To allow for sufficient migration of antigen-loaded DC to the draining lymph nodes, a population of intracellularly stained Sep- or Dep-transgenic T cells was adoptively transferred four days after immunization.

At 24 hours, both transferred populations could be detected in the lymph node in significant numbers but T cell division could not yet be observed (Figure 6, p41). Forward/side scatter analysis revealed that the onset of cell division already took place in the cluster-derived fraction. When being compared to free lymphocytes, cells dissociated from clusters contained an increased number of blasts, even though completed cell division could not yet be detected. For Dep-specific T cells, the first division, measured as CFSE fluorescence halving, could be observed at 30 hours in the cluster fraction but at this point not yet in the fraction of free lymphocytes (Figure 7, p42). Within the next 24 hours an increase in blasts occurred in both fractions and was accompanied by continuing cell division. Furthermore, at 36 hours after transfer the relative proportion of CFSE stained cells in the total population as well as in the blast fraction of dissociated lymphocytes was much higher than in the free ones and thus reflecting an enrichment of antigen-specific blasts within clusters. After 48 hours the population had divided four times, corresponding to an average division time of 4.5 hours. Now, the preponderance of blasts in clusters could not be found anymore and the relative proportion of CFSE-stained cells within the blast fraction was about twice as high in free cells as in the dissociated ones (24.9 ± 6.9% vs. 41.43 ± 9.2%). 72 hours after antigen encounter,

the proportion of CFSE-stained cells within the blast population started to decrease in the fraction of dissociated cells but continued to increase in the fraction of free lymphocytes.

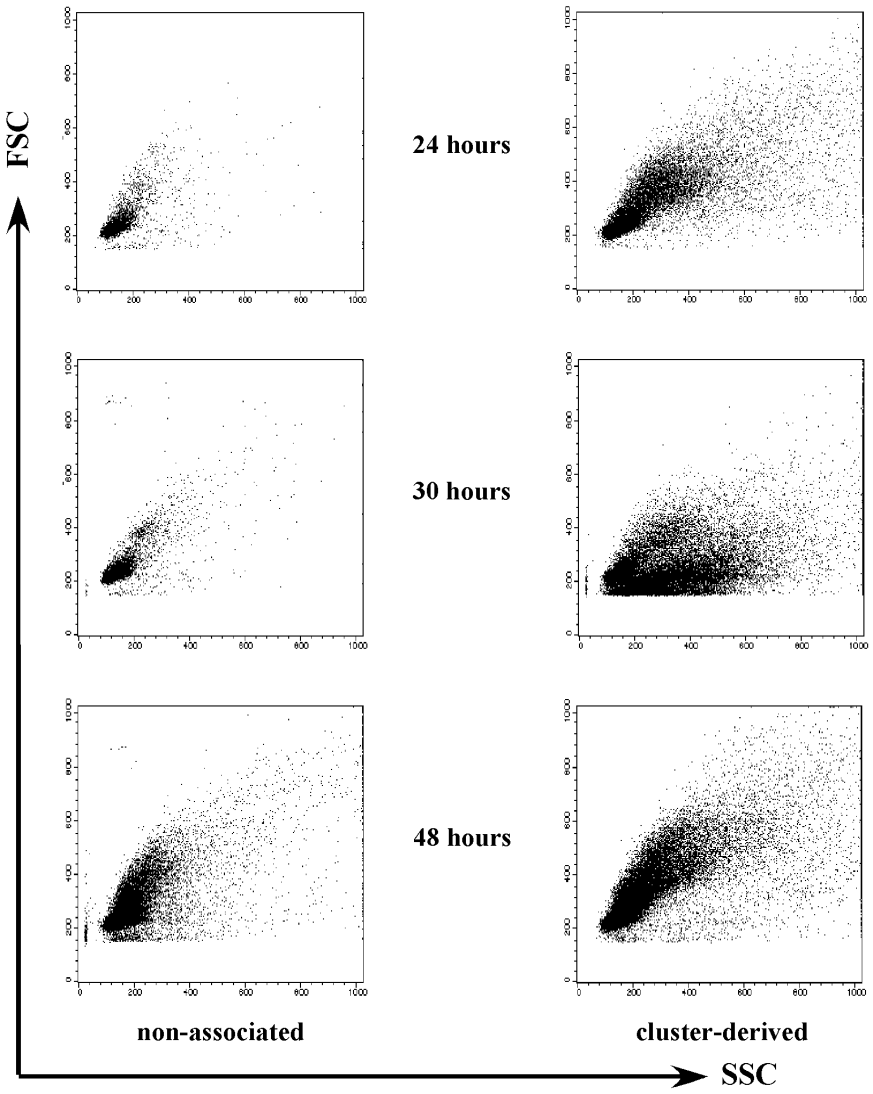

Figure 6 Blast formation is initiated in T cell/APC association. Mice were immunized with 50µg hCRP/CFA and subsequently received $5x10^6$ CFSE-labeled Sep- or Dep-specific T cells. Clusters were prepared at the indicated time points after transfer. Cluster-derived and non-associated cells were subjected to FACS analysis. Data of 50,000 cells total were collected. Here, one exemplary experiment with Dep-specific T cells is shown.

These results suggest an initial onset of proliferation while the T cells are still in contact with the APC, followed by an 'APC-independent' cell division. These findings are supported by the observation that the total number of clusters containing 3 or more CFSE-stained cells is highest during the early proliferation phase at the time of the first cell division and declines thereafter (Figure 3).

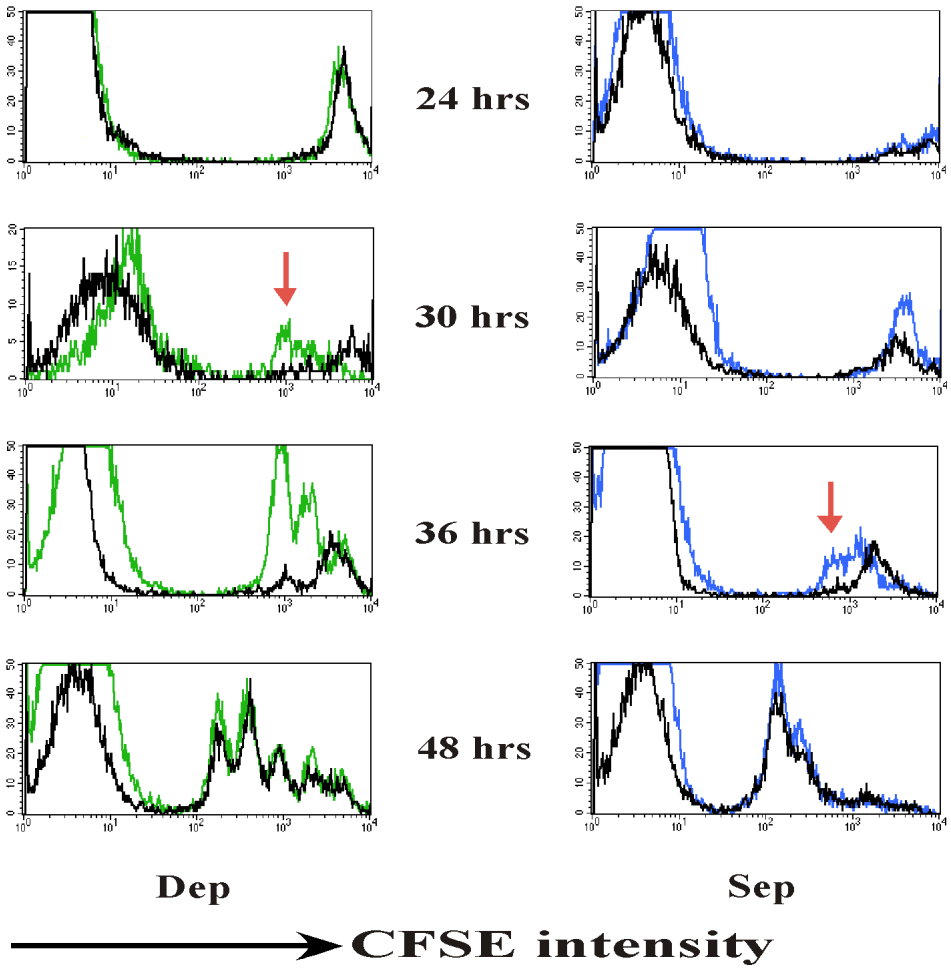

Figure 7 T cell proliferation is initiated in clusters. Mice were immunized with 50 µg hCRP/CFA and subsequently received $5x10^6$ CFSE-labeled Sep- or Dep-specific T cells. Clusters were prepared at the indicated time points after transfer. Cluster-derived and non-associated cells were subjected to FACS analysis. Data of 100,000 cells total were collected. Note that cell proliferation is initiated in the APC-associated fraction and that there is an epitope-dependent shift in timing of the first cell division. Coloured lines depict the APC-associated fraction; in black the respective non-associated cells. The analysis is restricted to large cells ("blasts").

Interestingly, even though T cells specific for a subdominant epitope of hCRP could also be localized in clusters at 24 hours, the first division of these cells lagged about 6 hours behind that of cells specific for the dominant epitope (Figure 7, red arrows). [In some experiments we could observe a very small proportion of cells undergoing cell division at 30 hours but here too, the majority of the T cell wave did not divide until 36 hours after transfer.] It should be pointed out that, – despite a later appearance of the first T cell division – Sep-specific T cells had also divided four times at 48 hours after transfer, amounting to an average division time of 3.5 hours. Apart from the 6-hour lag in the onset of proliferation, the same localization and

proliferation pattern was observed for both epitopes. Notably, proliferation of Sep-tg cells is less vigorous but the $t_{1/2}$ is shorter than that of lymphocytes specific for the dominant epitope, i.e. the average relative proportion of CFSE-labeled T cells found in the blast fraction was higher for Dep-tg cells than for Sep-tg ones [Dep: 41.4 ± 9.1% (free) versus 24.9 ± 6.9% (cluster-derived); Sep: 17.0 ± 3.2% (free) versus 14.0± 1.4% (cluster-derived)].
We wanted to further specify the onset of proliferation by analyzing first DNA replication. Would T cells specific for either the dominant or the subdominant epitope of hCRP be activated at the same time or would the delay in completion of the first cell division also be reflected in a delayed onset of transcription? For this approach we wanted to assess incorporation of bromodesoxyuridine (BrdU), a thymidine analogue. Unfortunately we could not obtain satisfactory results applying this method. Going through the procedure of cluster preparation and BrdU staining, which together took approximately 6 hours, appeared to be too stressful for the cells and FACS analysis was impossible to perform with the few cells left.

Having defined the time interval required for T cell activation for two epitopes in the hCRP model we wanted to know whether this finding would also apply to $CD4^+$ T cells of other specification. Thus we made use of the OVA/OT-II model, which also involves $CD4^+$ T cells restricted to the IA^b background as well as its $H2^d$–restricted equivalent, the OVA/DO11.10 model. Like hCRP-specific T cells, OVA-specific T lymphocytes could be detected within clusters at 24 hours after transfer in these two models as well (Figure 8, p44). Interestingly, completion of the first cell division was observed at 30 hours for both, OT-II- and DO11.10-specific T cells – the same time it was completed in the Dep-specific population – and again it was more pronounced in the cluster-associated fraction than in non-associated cells. At 48 hours cells of cluster-derived and non-associated fractions had undergone the same number of divisions and relative numbers of dividing cells were about equal [OT-II: 16.6% (free) versus 20.8% (cluster-derived); DO11.10: 6.9% (free) versus 8.3% (cluster-derived)]. This transition time mirrors the one observed in the hCRP model. These results indicate that the time frame and localization of T cell-activation of $CD4^+$ T cells specific for different dominant epitopes appears to be a constant parameter.

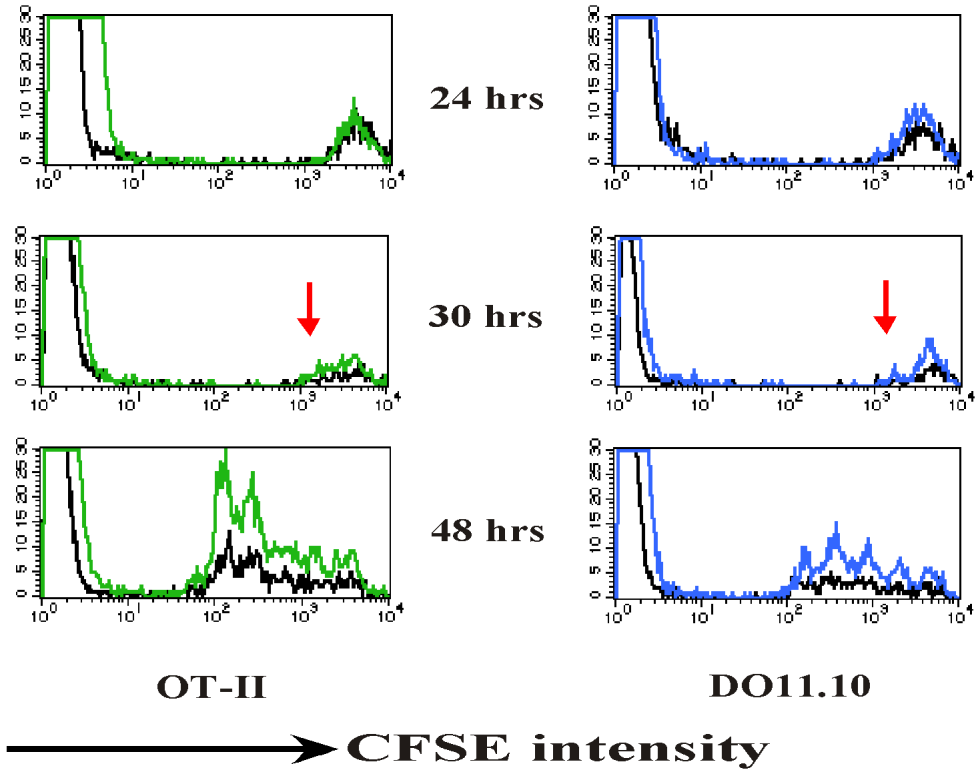

Figure 8 T cell proliferation is initiated in clusters. Mice were immunized with 50 µg OVA/CFA and subsequently received 5x10^6 CFSE-labeled OT-II- or DO11.10-tg T cells. Clusters were prepared at the indicated time points after transfer. Cluster-derived and non-associated cells were subjected to FACS analysis. Data of 100,000 cells total were collected. Note that cell proliferation is initiated in the APC-associated fraction. Coloured lines depict the APC-associated fraction; in black the respective non-associated cells. The analysis is restricted to large cells ("blasts").

The overall patterns of early antigen-specific T cell activation in four different CD4 models including two different MHC backgrounds (H2^b, H2^d) being the same, we asked whether T cells of the CD8 lineage would behave similarly. With the OT-I model available, BL/6 mice were immunized with 50-100 µg SIINFEKL or complete OVA. Four days later, 4-5x10^6 CFSE-labeled OT-I transgenic CD8$^+$ T cells were adoptively transferred. At 24-48 hours after transfer T cell/APC clusters were prepared as described and analyzed for proliferation.

Similar to the hCRP/Dep model, OT-I transgenic T cells had readily localized in clusters by 24 hours after transfer (Figure 9, p45). At 30 hours, the first T cell division had taken place, again in the cluster-derived fraction only. Proliferation continued during the following 18 hours but different to the CD4 models the percentage of CFSE-labeled cells in the cluster-derived fractions was remained above that of the non-associated fraction. Thus, CD4$^+$ and CD8$^+$ T cells behaved similarly with regard to compartmentalization but the average transition time of CD8$^+$ T cells apparently is longer.

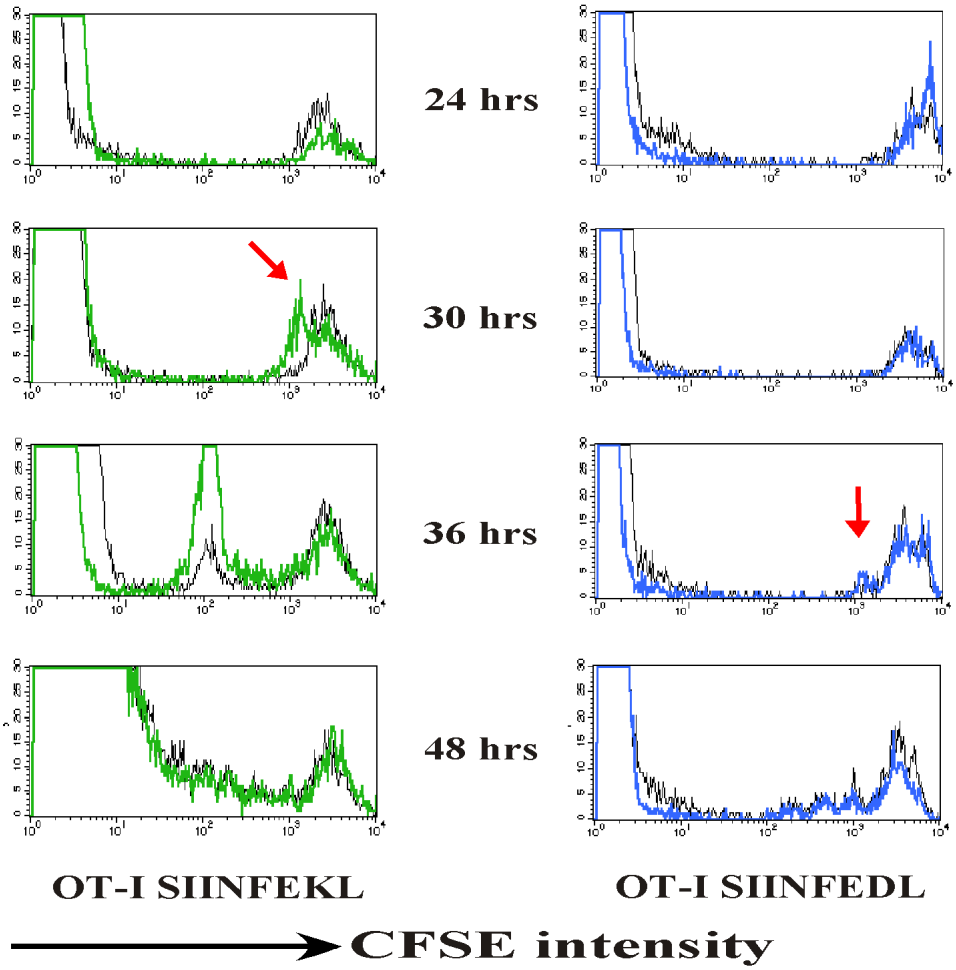

Figure 9 **Initiation of T cell proliferation is delayed for a weak agonist.** Mice were immunized with 50 µg SIINFEKL or SIINFEDL/CFA and subsequently received $5x10^6$ CFSE-labeled OT-I-tg T cells. Clusters were prepared at several time points after transfer. Cluster-derived and non-associated cells were subjected to FACS analysis. Data of 100,000 cells total were collected. Note that cell proliferation is initiated in the APC-associated fraction and that there is an epitope-dependent shift in timing of the first cell division. Coloured lines depict the APC-associated fraction; in black the respective non-associated cells. The analysis includes small lymphocytes as well as blasts.

Up to this point we could show that the onset of T cell proliferation in response to immunization turned indeed out to be a constant parameter: in models, in which lymphocytes responded to dominant epitopes, the first cell division had taken place 30 hours after transfer – irrespective of the T cell lineage or the MHC background. In the case of a subdominant epitope, a delay of roughly 6 hours could be observed. The OVA/OT-I model is well defined with regard to peptide derivatives: apart from the SIINFEKL peptide, which functions as a strong agonist, a number of weak agonists have been described (Jameson et al., 1993). Would a stimulus induced by such a weak agonist result in a proliferation history like that of the subdominant epitope or like that of dominant epitopes? Or would it result in a different

outcome of the immune response? We chose the OVA peptide$_{257-264}$ variant D7, which carries an aspartic acid instead of a lysine at position 7 and has been described to function as a weak agonist.

Again OT-1 transgenic cells could be detected in clusters 24 hours after transfer (Figure 9, p45). In contrast to clusters prepared from control animals that had been immunized with SIINFEKL, proliferation of the majority of antigen-specific T cells could not be detected before 36 hours after transfer in SIINFEDL-immunized mice. Three interesting observations could be made: First, at 48 hours, the mean fluorescence of dividing cells in the blast fraction was higher than that of controls indicating that they had undergone fewer divisions. Second, the relative and absolute numbers of cells taking part in the response were lower. Third, while in all other models specific lymphocytes found in cluster-derived and non-associated fractions had undergone the same number of divisions at 48 hours, in the SIINFEDL model cells in the APC-associated populations had undergone at least one more division than those in the non-associated one. Hence, a weak agonist delays the kinetics and reduces the extent of clonal expansion.

2.6 Microscopic Analysis of Early T cell Proliferation

While FACS analysis of CFSE dilution patterns allows determining the onset of proliferation as well as the time needed for each division with regard to the whole T cell population, microscopic analysis provides a means to directly correlate stages of T cell division to individual clusters as depicted in Figure 10 (p46). At 24 hours after transfer cells were still bright green and often small, even though blasts could already be detected. For Dep-specific lymphocytes, blast formation became more prominent at 30 hours, and first doublets could be observed. For Sep-specific T cells these stages appeared at 36 hours. With ongoing clonal expansion segregation was found within clusters and at 48 hours dim green dividing and small cells were also found cluster non-associated. At 72 hours most labeled cells had left clusters.

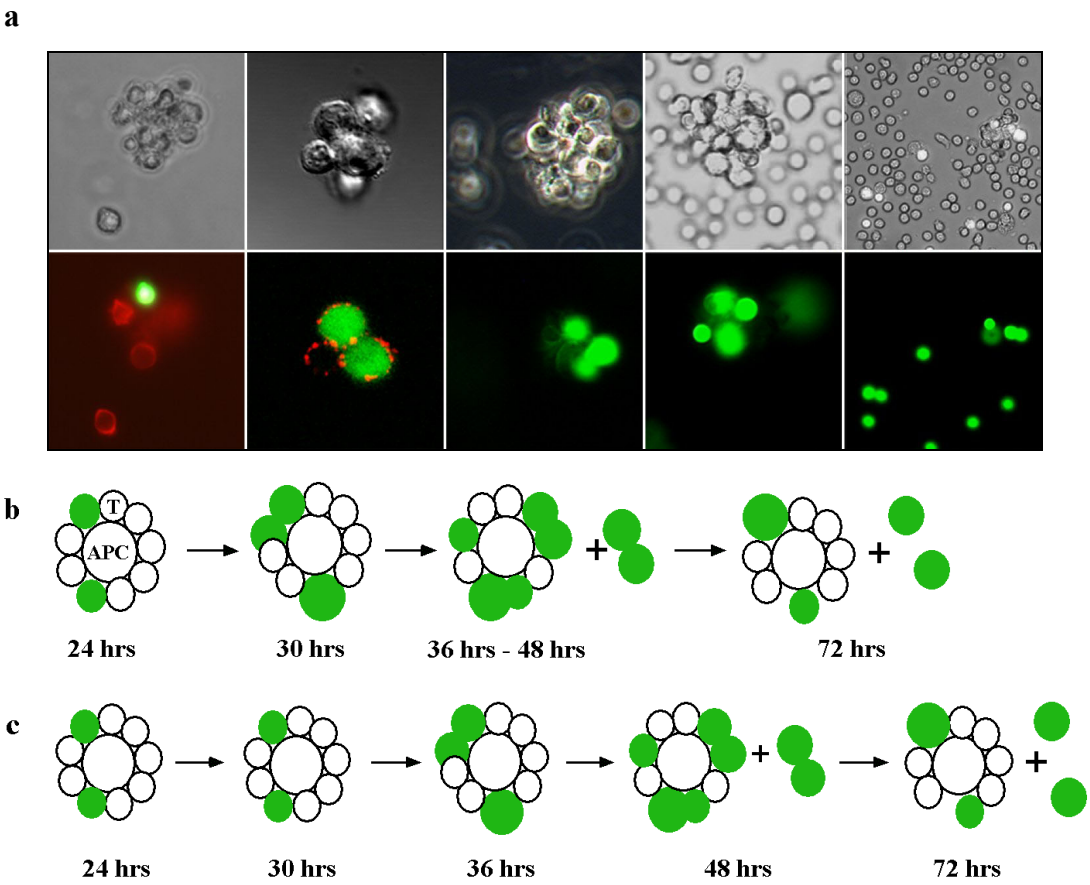

Figure 10 Microscopic analysis of early T cell proliferation. Different stages of T cell/APC interaction are depicted in (a). These interactions are shown schematically in order of their appearance over time for Dep- (b) and Sep-specific (c) T cells. Magnification: 400-fold, overview 250-fold.

2.7 Proliferation Kinetics are Antigen Dose-independent

What could the delayed onset of proliferation of T cells specific for the subdominant epitope be explained by? Can it possibly be attributed to distinct TCR affinities or to differential antigen-display? TCR affinities of both Sep- and Dep-specific T cell populations have not been precisely determined and therefore its correlation with delayed proliferation remains open. In *in vitro* experiments it was found that the T cell clone specific for the subdominant epitope (from which our TCR transgenic mice were generated) was more sensitive to stimulation than the Dep-specific one, i.e. lower peptide doses were required to reach equivalent levels of proliferation (Döffinger et al., 1997). On the other hand, presentation of the naturally processed dominant epitope has been shown to be 100-fold more efficient than that of the subdominant epitope (Klein et al., 1995). If lower peptide density was responsible for the delayed kinetics, a reduction in antigen dose should mimic such a situation.

We wanted to know whether lowering the amount of antigen available during the antigen-specific response would affect its outcome with regard to the onset, extent and duration of proliferation of the transferred cells. Mice were immunized with titrated amounts of hCRP. At several times after T cell transfer proliferation kinetics were performed (Figure 11).

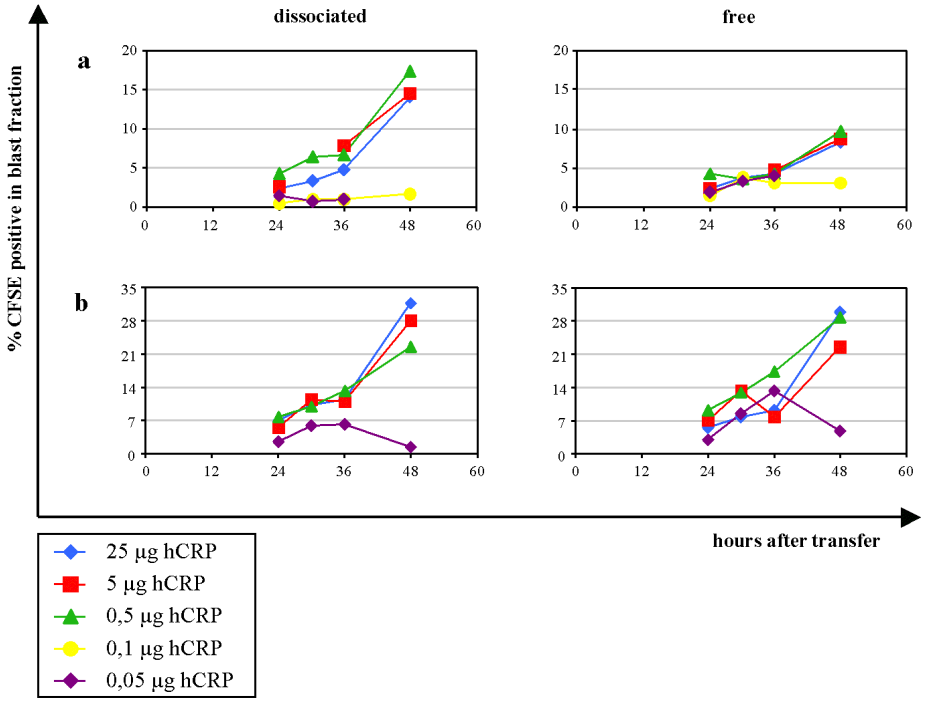

Figure 11 The T cell response to hCRP is sensitive to low antigen dose. Mice were immunized with titrated amounts of hCRP/CFA. Four days later 3-5x10^6 CFSE-labeled Dep- (a) or Sep-specific (b) T cells were adoptively transferred i.v. into the tail vein. Clusters were prepared at 24-48 hours after transfer, subjected to EDTA-treatment and assessed for CFSE halving patterns by FACS analysis. Data of 100,000 cells total were collected. Note that the extend of proliferation does not titrate; proliferation is, however, abolished when Ag-levels drop below a threshold of 0.1 µg/mouse.

Titrating hCRP from 50µg/mouse down to 0.5µg/mouse did not affect the time point of completion of the first cell division of either population. However, at antigen doses of 0.1µg/mouse or below proliferation could no longer be detected. Antigen-specific cells seem to temporarily accumulate in clusters, but do not receive stimulation strong enough to trigger T cell activation. Regarding the extent of proliferation also no dose-dependency could be observed. Proliferation of Sep-tg lymphocytes was always lower than that of Dep-tg ones. From these data we conclude that both T cell populations are highly sensitive to low antigen dose, whereas below a certain threshold of antigen available to APC no proliferation takes place. Above this threshold it appears not to matter how much antigen is applied: the outcome of the response does not alter.

2.8 Average 'Retention' Time in Clusters

Having defined T cell/APC clusters as functional units of initial T cell proliferation, the data obtained allows estimating the average 'retention' time of the T cell wave – the fate of individual cells cannot be determined with our method – in this compartment: The first T cells could be detected at 12-16 hours after transfer. After another 18-20 hours cluster-associated T cells had completed their first round of cell division, which then became independent of APC contact. Taking into consideration that at day 3-5 of an ongoing immune response highest numbers of antigen specific T cell are found in blood and spleen (data not shown), T cells are likely to be leaving the lymph node by 72 hours after transfer. Thus, the T cell wave needs approximately 32-36 hours to pass through the cluster 'compartment' from the time of first cell-cell interaction.

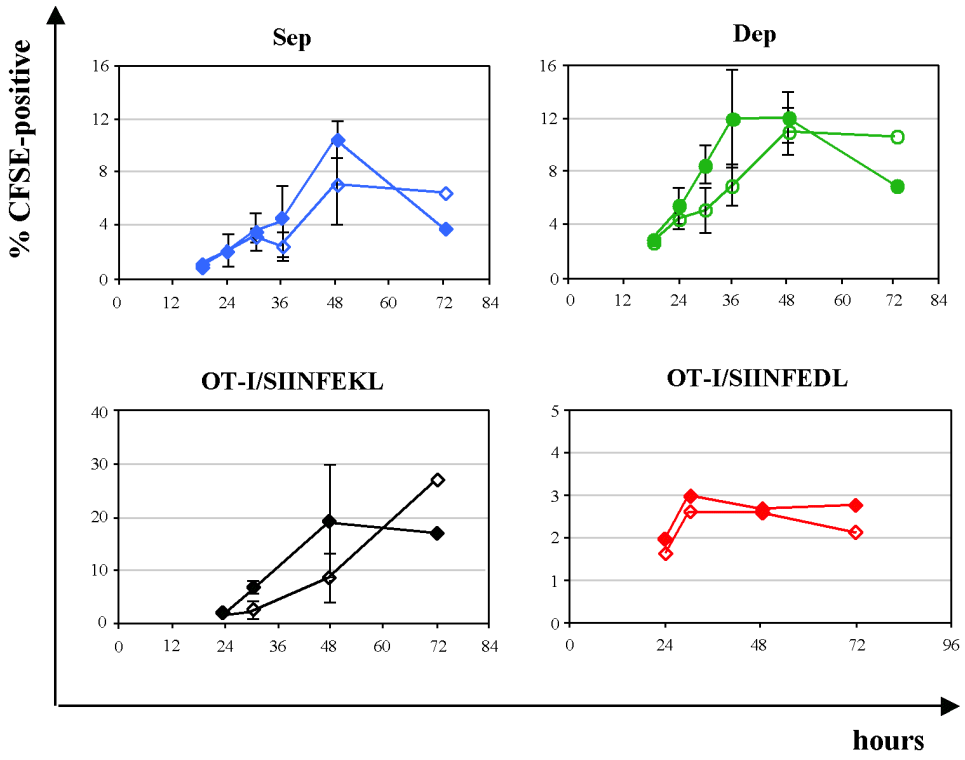

Figure 12 Average retention times in the cluster compartment. All cells (small lymphocytes and blasts) were included in the analysis. For Dep, Sep and OT-I/SIINFEKL the means and standard deviations of three independent experiments with 2 mice per time point are shown, for OT I/SIINFEDL data of one experiment are plotted. Open symbols represent the non-associated, closed symbols the cluster-derived fractions. Note that large error bars at later time points result from a widely varying extent of proliferation.

3 Concomitant Activation by two T cell Epitopes *in situ*

To date only little is known on whether epitope hierarchy influences clonal expansion and whether two adoptively co-transferred clonal T cell populations, which are specific for epitopes of the same protein, influence each other (Busch et al., 1998). Is the response to the subdominant epitope enhanced by a simultaneously occurring response to the dominant epitope, e.g. due to up-regulation of MHC class II or co-stimulators on APC (Sercarz et al., 1993)? Interleukins released by proliferating cells could create a local microenvironment, which facilitates the initiation of a response by cells directed towards subdominant epitopes that are located in the same cluster. Alternatively, the response could be suppressed because of competition for growth factors such as IL-2 or spatial limitations preventing the outgrowth of either population. This question could be important for the understanding of the etiology of autoimmune diseases, which is generally accompanied by the recognition of subdominant or cryptic self-epitopes, a process referred to as *'epitope spreading'* (Sercarz, 1998; Yu et al., 1996).

Previous work addressing the question of a mutual influence of two T cell populations specific for different epitopes of the same protein included adoptive transfer experiments into antigen-expressing hosts (Hommel, 1998). In these mice, hCRP is abundantly present (500 μM) and thus does not reflect an immune response, where antigen usually is temporally limited. In this model, within 24 hours transferred transgenic cells recognizing either epitope had disappeared from the blood and migrated to all peripheral lymph nodes as well as the spleen. After encountering antigen, the numbers of both Sep- and Dep-tg lymphocytes increased drastically, with peak numbers in the blood generally being found 4 to 5 days after injection. Peak numbers of T cells specific for the subdominant epitope were lower than those of lymphocytes specific for the dominant one. Nevertheless, the expansion and contraction pattern of the ongoing response was the same. These results were consistent with those of Busch et al. (1998), namely that T cell populations specific for dominant and subdominant epitopes expand with a similar kinetics but the responses differ in their magnitude: while dominant epitopes elicit large responses, subdominant epitopes elicit smaller ones. When we co-transferred both populations, an expansion pattern similar to that obtained from the single transfers could be observed. This implies that in a model where antigen is abundantly present (i.e. where both epitopes are presumably presented above threshold) there is no or only minor interference between the populations.

Limiting antigen availability would be expected to first result in a presentation below a critical threshold of the subdominant epitope while the dominant epitope would still be presented above. At such a dose the response should be most sensitive to the effects of intramolecular help. We therefore replaced the above transfer model by a 'more physiological' one to determine whether the two T cell populations behave similar or different under limiting antigen availability. In contrast to experiments performed by Busch et al., adoptive transfers in the hCRP model have the advantage that defined T cell populations can be followed.

3.1 Transient Antigen Supply

Non-transgenic C57BL/6 animals were injected i.v. with 4-5x10^6 transgenic T cells of either specificity or a mixture of both. This way it was possible to follow a pre-existing T cell population without overloading the system with T cells of one specificity. One day later 1-100 µg hCRP were applied intravenously to examine the course of the ongoing response not only in a time- but also in a dose-dependent fashion. If distributed equally in the whole body, the amount of 100 µg protein corresponds to approximately half of the basal concentrations of protein in the extracellular space found in hCRP-tg males that had been used in the transfer experiments described above. Since hCRP resources were limited, the injection of higher doses had to be renounced. The extent of T cell expansion was evaluated four days after antigen application by FACS analysis as the percentage of T cells carrying the transgenic Vß$^+$ chain within the CD4$^+$ population of the lymph node. Results are shown in Figure 13a+b (p52).

Upon single transfer both populations revealed a dose-dependent extend of proliferation. While cells specific for the dominant epitope (Dep) reached a value of 25% at the highest protein concentration and the titration curve was still increasing, cells specific for the subdominant epitope (Sep) made up only 10% of the lymphoid CD4 compartment and the expansion appeared to have almost reached the plateau phase. These results implicate that under antigen-limiting conditions dominant epitopes elicit larger responses than subdominant epitopes in this model.

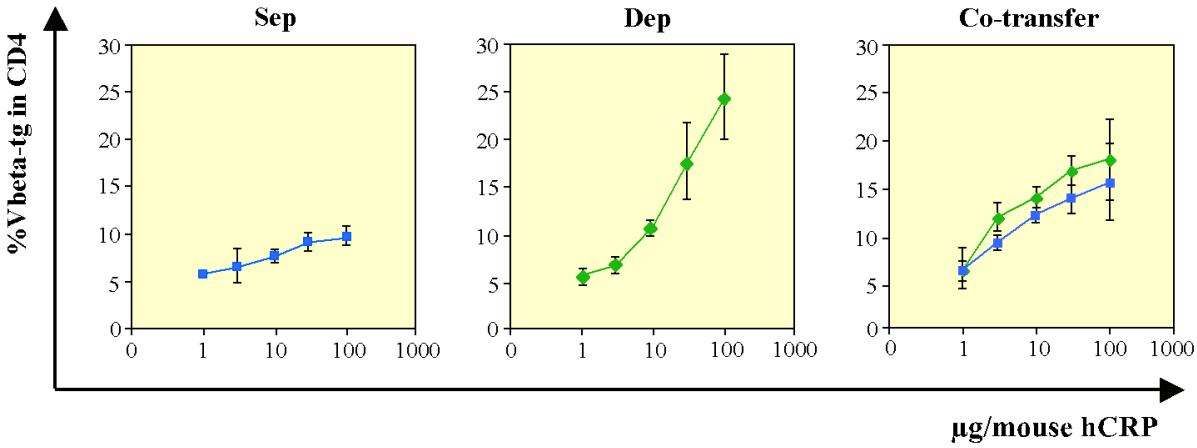

Figure 13 Dose response of Sep- and Dep-TCR-tg T cells upon single and co-transfer. Mice were adoptively transferred with $2x10^6$ Dep- or Sep-specific T cells or a mixture of both. One day later, titrated amounts of hCRP in PBS were injected i.v. into the tail vein. After four days mesenteric lymph nodes were harvested and analyzed for specific T cell proliferation. Data of 10,000 cells were collected. The results shown represent the means and standard deviation of three independent experiments, each including three mice. Note that both the response to the dominant and the subdominant epitope is altered upon co-transfer.

3.2 Altered Responses Upon Co-transfer

When both populations were co-transferred, the number of T lymphocytes responding to Sep was elevated for all hCRP concentrations (15% at 100µg hCRP). Also the shape of the curve of Dep-specific expansion reflected a pattern different to that of the single transfer: at low antigen concentrations there was a stronger response, but at high protein concentrations it now was significantly lower (Figure 13).

Taken together, these results document the phenomenon of ‘intramolecular help’ (Sercarz et al., 1993), which describes the dependency of one T cell population on another with respect to the threshold of activation. Thus, in the case of limiting hCRP availability this would mean that the proliferation capacity of T cells specific for the subdominant epitope is augmented by the simultaneously occurring response to the dominant epitope.

3.3 T cells of Different Specificity Co-localize in the Same Clusters

A necessary prerequisite for intramolecular help would be the co-localization of both populations in the same cluster. When antigen and T cells are injected i.v., the latter dilute too much to prepare clusters containing Ag-specific cells in numbers sufficient for analysis. Thus,

to ‘localize’ both T cell populations to the same APC, C57BL/6 mice were immunized in both hind footpads and a mixture of intracellularly labeled (CFSE and CMTMR) Sep- and Dep-specific T cells was adoptively transferred four days later. After two days, T cell/dendritic cell clusters were prepared from draining lymph nodes. Confocal microscopy allowed visualizing the co-localization of T cells specific for both epitopes.

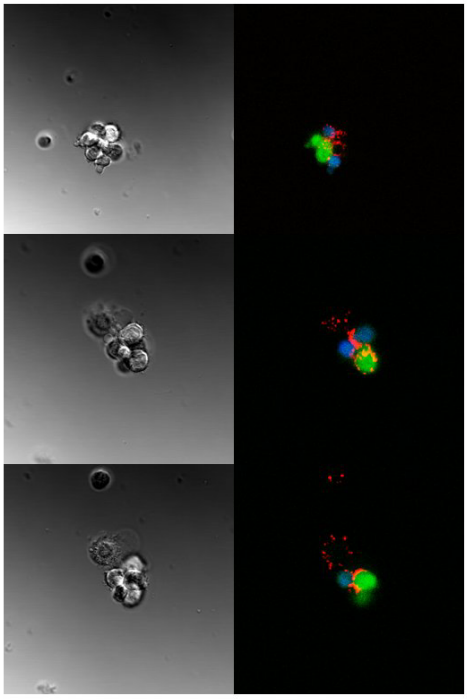

Figure 14 Co-localization of Dep- and Sep-specific T cells in T cell/APC clusters. Clusters were prepared at 48 hours after T cell transfer, fixed with 2% paraformaldehyde and stained for MHC class II-expression (red). Co-localization of Sep- (blue, CMTMR-labeled) and Dep-specific T cells (green, CFSE-labeled) was analyzed by confocal microscopy.

Cluster	Sep	Dep	Total No. of cells/cluster	Doublets
1	1	3	7	-
2	-	2	9	1*
3	2	2	5	1*
4	2	1	5	-
5	1	2	5	1*
6	-	2	5	1*

Table II Quantification of clusters containing transgenic T lymphocytes on day two after T cell transfer. Here, one exemplary experiment is shown. Note: To facilitate determination of labeled cells, only small clusters (≤15 cells) were included in the analysis. * Dep-tg only

Quantification of clusters containing fluorescent T cells (Table II) revealed that many of the cells taking part in cluster formation were of a transgenic phenotype. It could be shown that both cell types associated in the same clusters. Therefore, the pre-requisite for intramolecular help was given.

3.4 Prolonged Antigen Supply

To investigate whether there would be mutual influence upon co-transfer of both, Sep- and Dep-specific T cells, under limiting but prolonged antigen supply both populations were transferred simultaneously to Ag/CFA immunized C57BL/6 animals. As CFA acts as a depot antigen is released slowly. It therefore is available longer than upon i.v. injection but still temporarily limiting. Because no second intracellular dye with properties similar to those of CFSE was available, only one population could be labeled at a time. When clusters from co-transfers were prepared at different time points, no difference in the onset of proliferation could be observed (Figure 15). Only at 36 hours the extent of proliferation was a little higher in the single transfer controls.

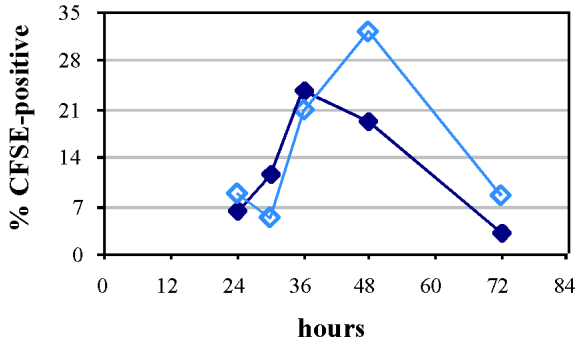

Figure 15 Upon co-transfer under transient but prolonged antigen availability T cells specific for the subdominant epitope behave as in single transfers. $5x10^6$ CFSE-labeled Sep-specific T cells alone (controls, dark blue) or a mixture of $5x10^6$ labeled Sep- and $5x10^6$ unlabeled Dep-specific T cells (light blue) were adoptively transferred to hCRP-immunized mice. Clusters were prepared at the indicated times, and proliferation of Sep-specific T cells was analyzed. Data of 100,000 events were collected.

Discussion

Successful induction of an immune response is not only determined by how 'foreign' the antigen is to the host, but also by the context in which it is presented, by its dose and by the duration of its presentation (Zinkernagel et a., 1997). These parameters may vary according to cellular environments in which an antigen is presented and the particular epitope recognized by T- or B cells. Clonal proliferation is an essential feature of the adaptive immune response. *In vivo* studies using T cell receptor transgenic models have provided new insight into this particular issue (Kearney et al., 1994; Ingulli et al., 1997).

Here, a novel methodological approach was introduced: We were able to isolate T cell/APC clusters directly from lymph nodes *ex vivo* and define them as functional units of T cell activation. Furthermore, labeled populations of T cells could be followed on their transit through this lymphoid microenvironment and it was possible to define several stages of the early proliferation phase. Thus, for the first time, we provide data bridging existing *in vivo* and *in vitro* studies. Several important findings could be made: First, it was shown that only recent immigrants segregate into such clusters. Second, the onset of proliferation was found to take place while the lymphocyte is in contact with an APC. Third, for dominant epitopes the time of completion of the first T cell division was determined to be a remarkably invariant parameter in different models, while T cells specific for a subdominant epitope and a weak agonist lagged behind in the onset of cell division. Fourth, in all models assessed the early APC-dependent proliferation phase was followed by an independent one and the average transition time for T cell populations specific for different antigens through the cluster compartment ranged between 32-36 hours from the time of first contact. Fifth, T cells specific for different epitopes of the same protein could be co-localized to the same APC.

Mitchison (1990) was the first to propose a role of multicellular T cell/DC clusters in mediating T helper activity and it has meanwhile been shown *in situ* that clusters of proliferating T cells are found in proximity of DC in T cell zones of secondary lymphoid organs after injection of superantigens (Luther et al., 1997) or allogenic cells (Kudo et al., 1997) and in an adoptive transfer model (Ingulli et al., 1997). Do the clusters isolated and described here represent the *ex vivo* correlate of the cell aggregations observed in the intact tissue *in vivo*? This contention is supported by the results described in this study and by previously published observations:

The group of Jenkins and colleagues adoptively transferred fluorescent dye-labeled, antigen-loaded DC and naive TCR transgenic $CD4^+$ cells specific for an OVA/$H2^d$ complex and used confocal microscopy to track their *in vivo* location. In lymph node sections they observed that OVA-specific T cells formed clusters around paracortical DC that were pulsed *in vitro* with antigen (Ingulli et al., 1997). In accordance with observations in these sections our aggregates were small, with approximately 10-20 cells clustered around each APC. Compared to untreated animals, we observed a 2-5-fold increase in numbers after immunization, thereby documenting a positive correlation between the occurrence of clusters and an ongoing immune response. Previous studies on multicellular clusters from the thymus, which are most likely analogous to those analyzed here, implied that the composition *in vivo* of thymus-derived lymphostromal cell interactions was retained during preparation and enrichment: When clusters were isolated from *in vitro* mixtures of thymuses of homozygous Thy1.1- and 1.2-congenic mice, T cells strictly segregated into individual aggregates according to their phenotype and clusters retained their phenotype during further enrichment. Furthermore, in chimeras containing varying proportions of Thy1.1 and 1.2 T cells, thymocytes of either phenotype segregated non-randomly into individual clusters (Kyewski et al., 1982).

Interestingly, when analyzing lymph node-derived aggregates, associated cells uniformly displayed low levels of CD62L as opposed to non-associated lymphocytes – irrespective of specific immunization. The CD62L molecule is the peripheral lymph node homing receptor used by naive circulating lymphocytes to enter lymph nodes via high endothelial venules (HEVs). Upon binding to its addressins and transmigration, CD62L is transiently down-regulated. Our observation not only suggests that cluster-associated lymphocytes represent recent immigrants, but also implies non-random cluster composition.

Selective composition is also reflected by the segregation of antigen-specific T lymphocytes into such aggregates: While at the time of first T cell division the overall proportion of transferred T cells was about 5%, a 4-fold enrichment of antigen-specific lymphocytes in individual T cell/APC clusters (> 3 cells/10-20) could be observed. At the same time the majority of clusters was devoid of any transferred T cells. Given this non-random distribution, it is highly unlikely that cluster formation occurs secondary to digestion and isolation. What could account for this conspicuous segregation? One possible explanation is the limitation of antigen-loaded APC. If this was true in our approach, immigrating T cells would have to 'search' for their specific APC. To date only little is known about the distribution of antigen-loaded DC within the paracortical region of the lymph node but in one report APC bearing a particular antigen appeared to be scarcely distributed within the T cell areas of the lymph

node (Inaba et al., 1998). Considering the mode of immunization as well as the dose dependency of this response, a limitation of antigen-bearing DC is unlikely to occur in the study presented here. Alternatively, antigen-loaded APC are not limiting, but those APC strategically localized at the entry site of T cells are preferentially seeded. A third possibility is that antigen-specific lymphocytes are limiting and therefore seed only few APC whereas other antigen-loaded APC remain empty. At present we cannot distinguish between these alternatives. In any case, segregation could either be the result of multiple independent seedings of an individual APC or of clonal expansion within a cluster. The observed sequence of events within a cluster – attachment of single small lymphocytes followed by blast formation and doublets – is in favour of the second alternative.

T cell/APC interaction is a necessary prerequisite for T cell activation, but to date little is known about the *in situ* localization of T lymphocytes at the early stages of proliferation. By using different transgenic transfer models and isolating clusters at various time points after T cell transfer it was possible to analyze this aspect: 12-16 hours after transfer the first T cells could be detected in the draining lymph nodes and started clustering around APC. Cell division was then initiated 18-20 hours later, while the T cells were still associated with DC. This early APC-dependent proliferation phase was followed by an APC-independent proliferation at later stages. The time needed for the 'T cell wave' from the initial APC contact to pass through the cluster 'compartment' was approximately 32-36 hours. It is important to note that the transition time was essentially constant for both T cell lineages (CD4 and CD8), in different transgenic models (hCRP, OVA) and on different MHC backgrounds ($H2^b$, $H2^d$). This time frame and the localization of T cell activation – as summarized in Figure 16 (p58) – agree well with the observations of Ingulli et al. (1997). They found that in their model transgenic OVA-specific $CD4^+$ T cells and Ag-pulsed DC accumulated to maximum level in the T cell areas of lymph node and spleen within one day of i.v. injection. The accumulation of T cell/DC clusters then slightly declined by 48 hours. Despite the disappearance of DC in the paracortical region, a dramatic increase of T cells in this area was found to take place during the following two days. Thus a DC-dependent phase was followed by a DC independent one. Furthermore, the group was able to correlate DC-T cell interaction with activation by staining for the production of IL-2.

The distinction of an APC-dependent versus APC-independent phase of the immune response has been recently addressed in several studies. Analyzing the activation requirements of naive CD4 T cells the group of Susan Swain concluded from their *in vitro* experiments that 'the

optimum effector generation depends on an initial two days of TCR stimulation followed by an additional two days of Ag-independent, cytokine-driven T cell expansion and differentiation' (Jelley-Gibbs et al., 2000). They also determined efficient antigen presentation to naive and effector T cells by professional APC to be in the range of two days. These *in vitro* data are well in line with our *in vivo* results. Indeed, the concept that $CD4^+$ T cells become activated in an Ag- and cell-cell contact-dependent fashion in the first two days of an adaptive immune response, which is then followed by an Ag-independent one, can possibly be extended to a third phase: proliferation of activated lymphocytes in the blood. Vasseur et al. (1999) assessed the distribution of cycling T lymphocytes in blood and lymphoid organs during the immune response to different antigens and found that the accumulation of Ag-specific T cells in the lymphoid compartment is only transient (1-2 days), with proliferating cells rapidly recirculating to the blood after activation. This also is in concordance with our finding that numbers of Ag-specific T lymphocytes in the lymph node start to decrease after two days.

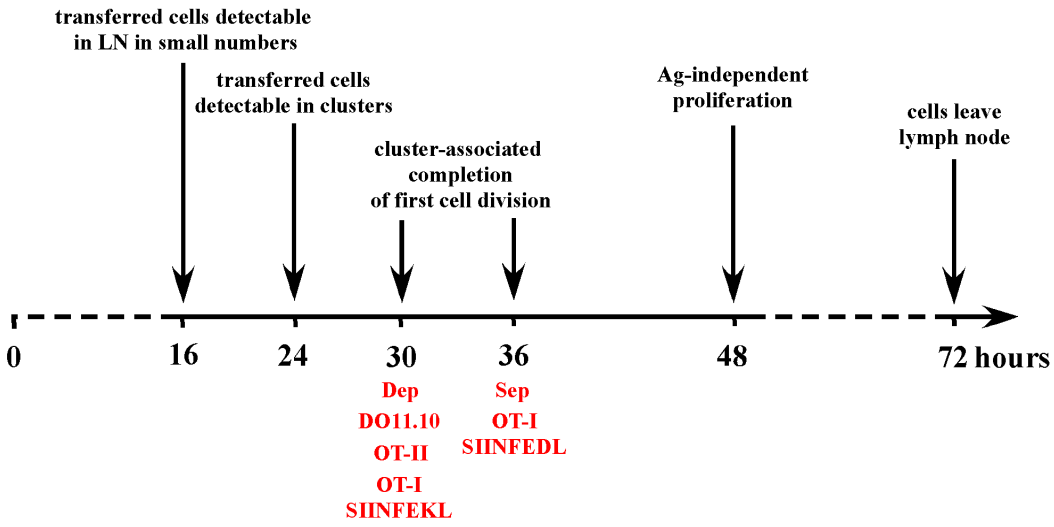

Figure 16 Schematic overview of T cell activation. Cellular events are shown above the time axis, and the time needed by each model to complete the first cell division below.

Two other groups addressed this issue for the CD8 T cell lineage. Van Stipdonk et al. (2001) as well as Kaech and Ahmed (2001) show that a 'brief' encounter with antigen is sufficient for naive $CD8^+$ cytotoxic T cells to start a 'program of proliferation and differentiation', independent of any further contact with antigen-bearing APC. Van Stipdonk et al. used engineered APC – a fibroblast cell line expressing the costimulatory molecule B7.1 and presenting a modified OVA peptide on MHC class I – and OT-I transgenic T cells to assess the degree to which the proliferation of naive cytotoxic T lymphocytes (CTL) depends on

activation stimuli. T cells were incubated with plate-bound APC and removed from these culture conditions at various time points to be placed in new, antigen-free cultures. As little as two hours of T cell/APC interaction turned out to be sufficient to fully commit the CTLs and drive them through several rounds of division. Kaech and Ahmed used a more sophisticated approach in that they combined *in vitro* and *in vivo* assays: naive $CD8^+$ T cells, which had been stimulated *in vitro* for 24 hours, were purified, intracellularly labeled with CFSE and transferred into normal BL/6 mice to monitor the proliferation and differentiation in the absence of antigen *in vivo*. Remarkably these cells underwent more than eight divisions, acquired full effector function and gave rise to memory cells.
Both studies confirm our finding that in the $CD8^+$ system, too, an early APC-dependent activation phase is followed by APC independent proliferation, while the time needed to complete the first cell division widely differs between the *in vitro* approach (van Stipdonk et al, 2001) and our *in vivo* data. Possible reasons for this difference shall be discussed elsewhere.

The finding that T cell activation is initiated in cell-cell contact comprises another important issue: the time needed by T cells from initial APC contact to proceed through their first division. Recognition of dominant epitopes resulted in completion of this first division by 30 hours after transfer, a surprisingly invariant parameter – independent of different model antigens (hCRP or OVA) or the MHC background ($H2^b$, $H2^d$). This observed lag of 18-20 hours from the initial T cell/APC contact to completion of the first round of cell division coincides with *in vivo* results from Gudmundsdottir et al. (1999) and *in vitro* data obtained by Jelley-Gibbs et al. (2001). Importantly, using an approach similar to ours – adoptive transfer of CFSE-labeled Ag-specific T cells and subsequent immunization – Gudmundsottir et al. could show that in the OVA/ DO11.10 TCR-tg model T cell proliferation was initiated at approximately 30 hours after immunization. When analyzing CFSE-dilution profiles of cell cultures, Jelley-Gibbs et al. observed that in their model naive $CD4^+$ T cells displayed a delay of 24 hours between initial TCR stimulation and the onset of proliferation. Furthermore, Iezzi et al. (1998) used influenza hemagglutinin (HA) specific T cells recognizing peptide 100-119 in the context of $I\text{-}E^d$ in *in vitro* assays under varying stimulatory culture conditions. From the data obtained the minimum contact time between TCR and surface bound peptide/MHC complexes for naive T cells to become committed to proliferation was determined to be in the range of approximately 20 hours. This minimum duration of interaction required for activation of naive T cells could be lowered to 6 hours

when mature DC were functioning as APC (Lanzavecchia et al., 1999). From these results in conjunction with our data, it can be proposed that proliferation of naive $CD4^+$ T cells is preceded by a prolonged phase of cell-cell contact and possibly TCR triggering.

In contrast, our observation of $CD8^+$ T lymphocytes also showing about 24 hours of APC contact before onset of proliferation is challenged by the *in vitro* data of van Stipdonk et al. (2001). 2 hours of interaction, as proposed in their model, appear to be strikingly short. In this context the work of Gunzer et al. (2000) should be delineated, who published an analysis on the duration, dynamics and frequency of T cell/DC interactions in an artificial extracellular collagen matrix: Using video microscopy, the group observed that T cell/DC clusters were small and T cells maintained vigorous movement upon interaction with cognate DC. The duration of T cell/APC interactions was short and transient (3-20 minutes for individual contacts) and resulted in a sequential sum of 2 hours total per 24 hours. When taking away the collagen matrix and keeping the cells in liquid culture only, an antigen-independent formation of large stable cell aggregates could be observed. Furthermore, proliferation in liquid culture turned out to be 4-fold more efficient than in the extracellular matrix. Thus, it is most likely that the short antigen-dependent phase found by von Stipdonk et al. could at least partially be accounted for by their experimental set-up, i.e. different stimulating conditions such as antigen density, T cell to APC ratio or stable aggregate formation.

To date we are unable to distinguish between prolonged or transient T cell/APC interactions during the observation period with our methodological approach, and Gunzer's findings do not necessarily contradict ours. On the other hand, in the matrix model T cell contacts to DC were shown to be both, specific and nonspecific and the presence of antigen did not alter the median interaction times as compared to antigen-independent interactions. This is in contrast to data implying that T cell activation is initiated in clusters and thus requires prolonged and stable cell-cell contacts, which occur as formation of the immunological synapse (Grakoui et al., 1999). Even though the clusters described in this study represent only a snap shot, the fact that they remain intact during isolation and enrichment requires cell-cell interactions of sufficient strength – as could be provided via stable and long-lasting synapse formation.

The requirements for successful T cell triggering entails the apparent paradox that MHC/complexes are of low affinity but nonetheless have to provide sustained cell-cell contact. Two models are presently tempting to reconcile available data on this issue: According to the single encounter model infrequent encounters between T lymphocytes and APC are stabilized by an adhesive mechanism to allow prolonged TCR triggering. Alternatively, the serial encounter model favours that effective T cell signaling is not a

continuous process, but acquired by repeated TCR engagement. T cells respond to the density of MHC-peptide complexes on several antigen-loaded DC but do not respond if the outcome of additive interactions remains below a certain threshold (reviewed in Lanzavecchia and Sallusto, 2001). Which model can our observations best be accommodated with?

The lymph node provides a microenvironment that differs from that of *in vitro* experiments using suspension culture or extracellular matrices: First, completely different from randomly arranged fibers of extracellular collagen matrices generated *in vitro,* lymph nodes have a much denser structure and contain larger numbers of cells. Second, the T cell area of resting lymph nodes (the so-called 'corridors' in the cortex) provides a cellular environment with the abundant collagen fibers being 'packed away' in stromal cells forming the reticular network and thus providing only little or no possibility of T cell-collagen contacts. The corridors are densely packed with cells and thus provide an ideal predisposition to foster cell-cell interactions between local APC and migrating T cells (Gretz et al., 1997). Interdigitating DC are the predominant APC in this area (Steinman, 1991). Even though they constitute only a small proportion of the overall cellularity of a lymph node (approximately 2%), their shape provides a large surface for contact as they become immobilized and line the fibroblastic reticular cells of the corridor space. After passing through this collagen-rich space, T cells enter the collagen-free parenchyma. Third, even though migrating T cells continuously follow chemokine gradients, the key chemokine receptors necessary for migration, such as SLC, are likely to be desensitized upon entry through HEVs (Ebnet et al., 1996). Thus, due to its 'micro-architecture' the lymph node represents an environment, which should favour prolonged rather than transient cell-cell interactions.

Apart from the appropriate microenvironment, extended T cell/APC interaction – as probably reflected in the delay from initial T cell/APC contact to the onset of proliferation observed in our models – would also require a certain stability of MHC-peptide complexes. If their half-life ($t_{1/2}$) was short (in the range of minutes to few hours) and they were lost from the cell surface quickly, this would abrogate cell-cell contact. Interestingly, the $t_{1/2}$ of MHC-peptide complexes was found to be dependent on the microenvironment and the APC subtype but independent of the combination of MHC and peptide. Thus, it appears to be an invariant parameter that is determined by the turnover of MHC-peptide complexes on the cell surface and not by the turnover of APC themselves (Röttinger, 2000). For Langerhans cells and immature APC of the spleen a $t_{1/2}$ of between four to six hours could be determined while in non-activated mesenteric lymph nodes they ranged from seven to ten hours. Moreover, the ability to present MHC-antigen complexes to T cells could be strongly improved upon

combined antigen-CFA application: CFA induces the maturation of DC and therefore increases the proportion of mature DC in the draining lymph nodes. With maturation they lose their ability of efficient antigen-uptake and processing but convert to very effective antigen presenters (Guèry et al., 1997). When antigen was applied in combination with CFA, the $t_{1/2}$ of MHC-peptide complexes presented on the cell-surface increased by a factor of 3-6 to more than 30 hours. This $t_{1/2}$ of MHC-peptide of 30 hours on mature DC would provide for prolonged antigen availability for activation and is concordant with the mean interaction time of T cells with APC *in vivo*.
Hence, both the lymphatic microenvironment as well as the prolonged $t_{1/2}$ of MHC-peptide complexes are more easily reconciled with the single encounter model.

Interestingly, even though in all models analyzed transferred T cells could be localized in clusters at the same time, the onset of proliferation of T lymphocytes specific for a subdominant epitope in the hCRP model and that of T cells responding to a weak agonist in the OVA/OT-I TCR-tg model lagged behind that of cells specific for the corresponding dominant epitopes. Additionally, T cells specific for both, the dominant and subdominant epitope of hCRP, had undergone equal numbers of divisions at 48 hours after transfer – despite the delayed onset of proliferation of Sep- specific lymphocytes. How can these findings be explained? Two possible explanations have to be considered: lower epitope density and, alternatively, distinct TCR affinities. Epitope dominance is defined as more efficient presentation compared to subdominant or cryptic epitopes. In the hCRP model antigen-presentation of the naturally processed subdominant epitope is 100-fold weaker than of the dominant one (Klein et al., 1995). Dominance of one epitope versus subdominance of the other may be due to different mechanisms (Döffinger et al., 1997): First, the processing of hCRP may take place in a manner protecting the dominant epitope while preferentially degrading the subdominant one. Second, the dominant epitope may bind much stronger to IA^b and thus prevents a more efficient presentation of the subdominant epitope. Third, immunodominance could be the result of quantitative and qualitative differences in the T cell repertoire. The possibility of a higher T cell precursor frequency specific for the dominant epitope can be excluded in the experiments presented here as equal numbers of transgenic T cells were transferred. If the dominant epitope was presented more efficiently than the subdominant one, this could account for an earlier onset of proliferation of T lymphocytes specific for the dominant epitope, whereas a higher affinity of T cells specific for the subdominant epitope could be an explanation for increased proliferative activity.

If the lag in the onset of T cells specific for a subdominant epitope can most likely be attributed to its lower presentation, what could account for that of a weak agonist? Weak or partial agonists are variants of antigenic MHC-peptide ligands that have altered capabilities to induce T cell responses. The ability of MHC-peptide complexes to exhibit biological activity correlates with their binding affinity for the TCR and weak agonists have a faster off rate than full agonists (Germain and Stefanova, 1999; Jameson and Bevan, 1995). The attempt to explain the influence of small differences in off rates of the TCR on the time required by T cells to become committed to activation resulted in two models (reviewed in Germain and Stefanova, 1999): the kinetic proofreading and the kinetic discrimination models. Both predict that ligands with fast off rates permit very transient TCR interaction only, thereby inducing very early but not late activation events. This way, the minimum time required to complete formation of a fully competent signaling complex would enable the TCR to discriminate between high and low-affinity ligands. An approach to link this discrimination to defined intracellular events was recently published by Rosette et al. (2001). They assessed the OT-I T cell response towards a low- versus a high affinity TCR ligand with regard to differences in early and late activation events. Using conventional APC or tetrameric forms of the antigen, the authors could show that the differences in T cell activation by either OVAp or its weak agonist variant G4 were not due to the steady-state occupancy of the TCR but rather to kinetic parameters such as the duration of TCR/MHC-peptide interaction. Furthermore the kinetics of the T cell response did not correlate with alternative activation pathways. Both OVAp and G4 were able to induce maximal CD69 up-regulation *in vitro,* but 1000- to 10000-fold higher doses of G4 were required to reach equivalent responses. The expression of CD69 was similar at 24 hours but up-regulation in response to G4 was found to lag at least 5 hours behind that of OVAp. CFSE dilution assays also revealed a similar lag in the proliferative response. These *in vitro* results are in perfect concordance with our *in vivo* observation. Based on their findings, the authors proposed a revised model of kinetic proofreading in which activation by low-affinity ligands leads to full responsiveness by 'trickling through to less reversible downstream signaling intermediates'. A possible candidate to act as a 'counter' for productive TCR/ligand engagements is phosphorylated c-Jun, which was shown to slowly accumulate over time.

To place our findings in the context of current models on the dynamics of T cell activation, a revised version is proposed: If – upon recognition of a weak agonist – individual TCR/ligand interactions are more transient than those with strong agonists, prolonged signaling will be

required to accumulate a critical number of effective intracellular 'counts' and result in a positive signal. This can be achieved by either of two possibilities: increasing the absolute number of interactions or extending the individual interaction time. Including the serial encounter model in these considerations, an increase in the number of interactions can result from more numerous individual TCR/MHC-peptide contacts on the same APC but also from multiple (serial) encounters with different APC. It therefore is possible that accumulation of intracellular 'counters' above a critical threshold induces a 'stop' signal, allowing for a stable and prolonged cell-cell contact in form of the immunological synapse. This in turn elicits late activation events and the lymphocyte can proceed through cell cycle. In the case of subdominants epitopes one would predict that to accumulate enough positive counts the number of interactions also has to be increased – not because of more transient interactions compared to dominant epitopes but because of lower epitope density. Hence a longer interaction time would be required. This way both, serial encounter as well as stable synapse formation could be combined in one model.

The hCRP model allowed us to address another important feature of the adaptive immune response, the mutual influence of T cell populations specific for epitopes of the same protein. The influence of epitope hierarchy on clonal expansion could be important for the understanding of the etiology of autoimmune diseases, which are generally accompanied by the recognition of new self-epitopes, a process referred to as *'epitope spreading'*. 'Epitope' or 'determinant spreading' was first introduced by Lehmann et al. (1992) to describe the process in which an immune response directed to a single self-peptide can spread to include other determinants on the same auto-antigen (*intra*molecular spreading) as well as on other self-antigens (*inter*molecular spreading). While dominant epitopes elicit large immune responses, subdominant ones elicit smaller ones (Sercarz, 1998). This was also true in the hCRP model. Expansion of polyclonal T cell populations with respect to epitope hierarchy and MHC-peptide competition has recently been subjected to detailed analysis by several groups. Busch et al. (1998) showed that distinct endogenous T cell populations specific for *Listeria monocytogenes*-derived epitopes expand, contract and enter the memory compartment at the same time. Adoptive transfer experiments have the advantage that defined T cell populations can be followed through the response. Therefore the hCRP model provided a useful tool to approach the question of clonal influence and expansion under different antigen availability as depicted in Figure 17:

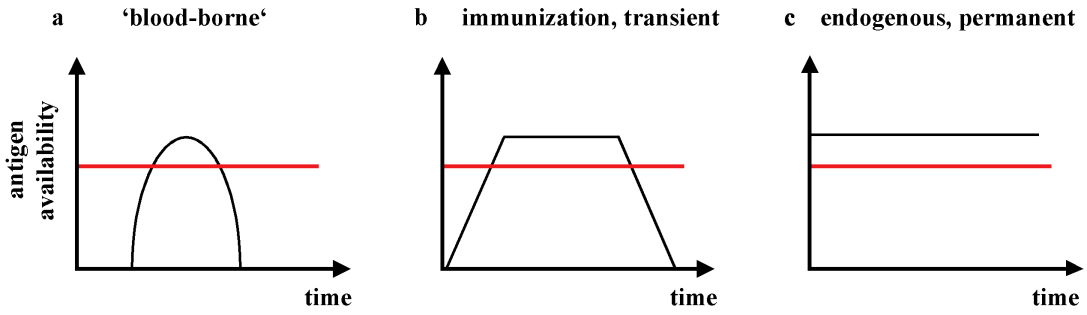

Figure 17 Antigen availability in different application modes. (a) reflects the 'natural' presence of hCRP as found during an acute phase: antigen is applied exogenously by i.v injection and the amount necessary to induce a T cell response (threshold indicated by red line) is only transiently available. (b) Upon immunization with CFA, the antigen availability is also limited but remains above threshold level for a prolonged period of time. (c) In hCRP transgenic animals, antigen is expressed endogenously and levels are permanently above threshold.

Data obtained from transfers under non-limiting antigen availability (Figure 17c) were in line with those of Busch et al. (1998), in that responses of T cell populations specific for dominant and subdominant epitopes expanded with a similar kinetics but differed in their magnitude: while dominant epitopes elicited large responses, subdominant epitopes elicited smaller ones. Upon co-transfer of both populations, an expansion pattern similar to that obtained from the single transfers could be observed (Hommel, 1998). This implies that in a model where antigen is abundantly present and therefore both epitopes are presented well above a critical threshold, there is no or only minor interference between the populations. In contrast, limited antigen availability would be expected to alter the overall epitope presentation and hence the outcome of clonal expansion: If antigen was available only transiently, presentation of the subdominant epitope would fall below the threshold level necessary to elicit an adaptive immune response more quickly or the epitope could even become cryptic. Dominant epitopes would be presented at lower levels as well but still high enough to remain above the critical threshold. On one hand, this could account for a reduced or aborted response of T lymphocytes specific for the subdominant epitope, on the other hand co-transfer of both populations could result in intramolecular help, thus elevating presentation of the subdominant epitope above threshold (see Figure 18, p66).

Upon co-transfers under limiting but prolonged conditions (Figure 17b) no differences in the time frame or extend of proliferation compared to single transfers could be observed, indicating that both epitopes were still presented above threshold. In contrast, short and transient antigen availability (Figure 17a) resulted in both populations showing the same extent of proliferation upon co-transfer while in single transfers Dep-specific T cells

proliferated much more vigorously than those specific for the subdominant epitope. This implicated intramolecular help was taking place.

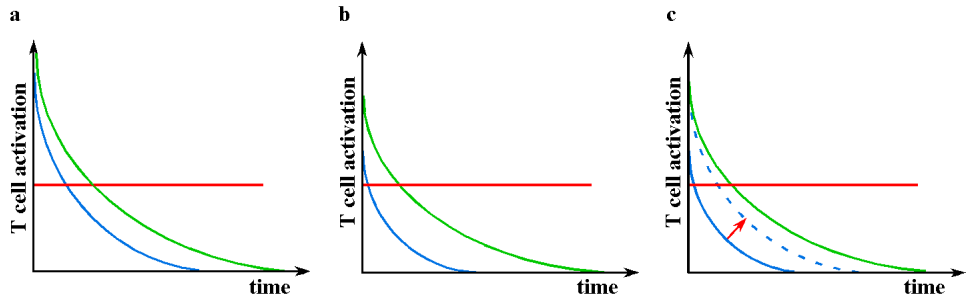

Figure 18 Antigen presentation and intramolecular help. In a situation when antigen is prolonged and transiently available, both a dominant (green) and a subdominant epitope (blue) are presented above a critical threshold (a). Over time, levels decrease until presentation is no longer sufficient to elicit any responses. Is antigen only short and transiently available (b), the number of MHC-subdominant epitope complexes is just above or even below the critical threshold, hence inducing only weak responses. Upon intramolecular help (c), the response to the subdominant epitope is augmented (dotted line).

It is suggested that upon T cell activation, the APC that is engaged in antigen recognition receives signals up-regulating the expression of molecules such as MHC class II or co-stimulators (Sercarz et al., 1993). This in turn could lead to an increased presentation of the subdominant epitope and thus provide the possibility of stronger T cell triggering. Alternatively cytokines locally secreted by an activated T lymphocyte may stimulate a neighbouring T cell on the same APC, specific for a subdominant or cryptic epitope as proposed by Mitchison (1990). In the case of experimental autoimmune encephalomyelitis (EAE), Yu et al. (1996) found a predictable pattern in which new determinants are recognized, and they also showed an invariant relationship between relapse and progression of the disease and recognition of new epitopes. Both could be accounted for by an increased response to the subdominant epitope as observed in our experiments. While 'functional' three-cell-type clusters were proposed over ten years ago by Mitchison (1990) and many groups have provided indirect evidence for their existence, here we show that the prerequisite for intramolecular help was given.

While the enhanced proliferative capacity of Sep-specific T cells is likely to be the result of intramolecular help, how can the finding be explained that at higher antigen doses Dep-specific T cells peaked about half as high in co-transfer experiments as they did upon transfer on their own? Maybe not all of epitope dominance can be explained in terms of affinity for MHC and peptide loading as proposed by Chen et al. (2000). There is evidence suggesting

that T cells responding to one antigen can actively interfere in a competitive fashion with T cells responding to another (Wolpert et al., 1998; Grufman et al., 1999). Kedl et al. (2000) used a model in which endogenous OVA-specific T cells competed with adoptively transferred OT-1 TCR-transgenic cells for a dominant and a subdominant OVA epitope *in vivo*. Transfer of OT-I cells, which are of high affinity for the dominant epitope, successfully inhibited the endogenous responses to both epitopes – provided they were presented on the same APC. Inhibition could be alleviated by transfer of large numbers of antigen-pulsed APC. Thus, high affinity T cells efficiently compete for space at the DC and prevent low affinity cells from acquiring enough TCR/MHC-peptide interactions. Interestingly, in the absence of OT-1 cells little inhibition of the response against either epitope could be observed. The authors contributed this to the prediction that low affinity endogenous T cells are inefficient at competing with T cells of other specificities. In context of these findings, our data on co-transfer experiments under short and transient antigen availability suggest the following scenario: Proliferation of Dep-specific lymphocytes results in intramolecular help, thereby up-regulating complexes of MHC-subdominant epitope and reinforcing clonal expansion of Sep-specific cells. If the latter are of a higher affinity for their epitope, they will now compete with Dep-specific lymphocytes for space at the hCRP presenting cells, thus impeding the 'helper' population. When antigen is available above critical threshold, this effect is abrogated as observed.

The TCR-affinities of both lymphocyte populations have not yet been defined but here we have shown that they are very sensitive to low antigen dose: at hCRP concentrations from 50 µg to as little as 0.5 µg/mouse neither differences in the onset nor in the extent of proliferation over time could be observed. Regarding the subdominance of one hCRP epitope, this implies rather high TCR affinity. Surprisingly, at concentrations below 0.5 µg/mouse, proliferation could not be detected anymore. What could account for these findings? First, we do not know whether the amount of antigen arriving in the lymph nodes directly correlates with the dose injected into the footpad. Second, it is unknown whether reduction of the applied antigen is reflected in a reduction of the overall MHC-peptide density on each individual APC or whether the density remains the same and the absolute number of cells presenting antigen is reduced (Figure 19).

A lower epitope density would be likely to result in delaying the onset of proliferation, as the previously dominant epitope would now be presented at 'suboptimal' levels. The density of the previously subdominant epitope may now be below the threshold required for T cell commitment, thus completely abolishing clonal expansion. On the other hand, if the number

of APC became limiting but the individual epitope density remained the same, one may expect a shift in the extend of proliferation. Since neither a delay in the onset of proliferation nor a titration of clonal proliferation was observed, other parameters must influence the antigen dose dependency.

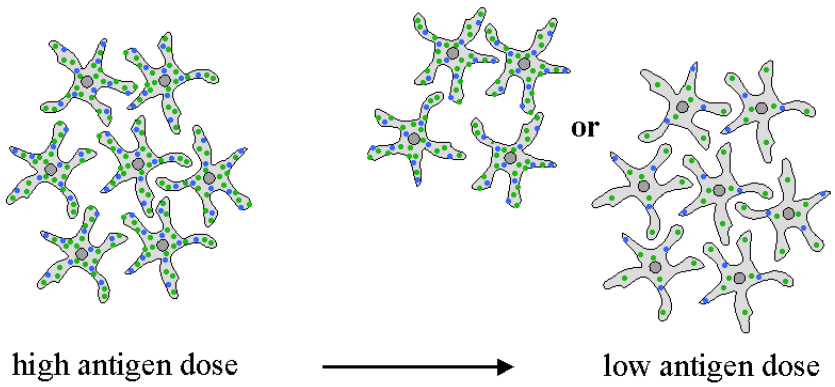

Figure 19 Antigen distribution at high and low antigen doses. Reduction of the amount of antigen used for immunization may result in a decrease of MHC-peptide complexes on each individual APC (while the number of APC involved in antigen presentation does not alter) or in a decrease of the overall number of APC, each expressing a number of complexes on their surface that is equivalent to those at high antigen dose.

Outlook

Upon T cell/DC interaction T cells of either the same or of different specificities are brought into close proximity and this provides a prerequisite not only for linked help but also for immune deviation as well as the interaction with regulatory T cells (T_{reg}). In particular the existence of T_{reg} cells and their mode of action has recently received a lot of attention. It has been a great controversy among immunologists, especially due to the ambiguity in the molecular basis of suppression. With growing experimental evidence such as the spontaneous occurrence of various autoimmune diseases upon depletion of certain $CD4^+$ subsets (reviewed by Shevach, 2000), their existence is meanwhile widely accepted and they provide one means by which self-reactive T cells are controlled in the periphery. The ability to isolate antigen-specific T cells at various stages of activation provides a possible means to isolate T_{reg} *ex vivo* at their site of action.

Furthermore, cluster isolation provides an approach to further dissect the early phase of clonal expansion and address issues such the role of synapse formation more closely: In the current model proposed by Dustin et al. (2001) synapse formation is regulated by

microenvironmental factors to restrict the site of primary immune responses and to provide a control for the duration of T cell/APC interactions. As all data on the establishment of synapse formation were obtained from in *vitro culture*, our approach would offer the possibility not only to verify synapse formation *in vivo* but also to analyze the time-course of synapse resolution *ex vivo*. By combining our approach with video microscope analysis we should be able to determine the stability and therefore the duration of cell-cell interaction after cluster isolation. Finally, isolating T cell/APC clusters at defined time points should allow subjecting cluster-derived, antigen-specific lymphocytes to an extended genetic analysis of T cell activation. For example, gene expression patterns of early and late activation events could be assessed by gene arrays. This way the *in vivo* relevance of *in vitro* observations could be validated.

Summary (German)

Zell-Zell-Interaktionen *in situ* während der frühen Phase der T-Zellaktivierung

Die erfolgreiche Induktion der adaptiven Immunantwort ist von der Antigendosis, der Dauer der Antigenpräsentation sowie der zellulären Mikroumgebung abhängig. Diese Parameter können sich je nach Epitop, das von T- oder B-Zellen erkannt wird, voneinander unterscheiden. Das *in situ* Verhalten klonaler T-Zellpopulationen ist erst in den letzten Jahren der präzisen Analyse zugänglich geworden. So konnte gezeigt werden, daß Cluster proliferierender Zellen um dendritische Zellen (DZ) herum lokalisiert sind, die die MHC Klasse II-exprimierenden antigen-präsentierenden Zellen (APZ) in der parakortikalen Region des Lymphknotens ausmachen. Eingewanderte T-Zellen interagieren mit DZ und erscheinen aggregiert. Die Isolation solcher Aggregate aus dem Thymus ist bereits beschrieben worden. Hier wurde diese Methode adaptiert und modifiziert, und es war zum ersten Mal möglich, eine Verbindung zwischen veröffentlichten *in vitro* und *in situ* Studien herzustellen. Das Ziel dieser Arbeit war es, T-Zell/APZ Cluster aus Lymphknoten von Mäusen zu isolieren und als Kompartment der T-Zellaktivierung zu charakterisieren. Die frühe Phase der klonalen T-Zellexpansion im Hinblick auf Lokalisation und Kinetik wurde analysiert.

Isolation und Charakterisierung von Clustern

Cluster wurden aus drainierenden Lymphknoten von CFA-immunisierten Tieren oder Mäusen, die mit Antigen/CFA immunisiert worden waren und im Anschluß antigen-spezifische T-Zellen erhalten hatten, isoliert. Die Lymphknoten wurden zu verschiedenen Zeiten herausgenommen, das Gewebe mit Kollagenase/Dispase verdaut und die Cluster anschließend über einen 1 x g Gradienten angereichert. DZ konnten über den Oberflächenmarker CD11c identifiziert werden und fungierten in etwa 70% der Cluster als zentrale Zelle. Die durchschnittliche Anzahl der T-Zellen pro Cluster lag bei etwa 10-20. Die Clusterbildung an sich war nicht strikt antigenabhängig – auch aus mesenterialen Lymphknoten unbehandelter Tiere konnten sie in kleiner Anzahl gewonnen werden (wobei hier eine natürliche Infektion nicht ausgeschlossen werden kann) –, allerdings stieg die Anzahl nach Immunisierung stark an.

Phänotypische Analyse der cluster-assoziierten T-Zellen

Interessanterweise ging die Clusterbildung mit einer Segregation erst kürzlich in den Lymphknoten eingewanderten T-Zellen einher: Wie anhand der Färbung des Homing-markers CD62L, der auf naiven, rezirkulierenden Lymphozyten exprimiert und bei Eintritt in den Lymphknoten herunterreguliert wird, deutlich wurde, konnte seine verminderte Expression nur auf cluster-assoziierten, nicht jedoch auf freien T-Zellen nachgewiesen werden. In Mäusen, die mit Antigen immunisiert worden waren und einen T-Zelltransfer erhalten hatten, konnte außerdem eine Segregation antigenspezifischer Cluster gezeigt werden. Während der Gesamtanteil an transferierten Zellen relativ gering war, lag die Anzahl der Cluster, die mehrere dieser Zellen enthielten, hoch. Dies war besonders zum Zeitpunkt der ersten Teilung sehr ausgeprägt. Desweiteren konnte die Aktivierung von T-Zellen direkt mit der Notwendigkeit der Zell-Zell-Interaktion korreliert werden, denn nur T-Lymphozyten aus T-Zell/APZ Aggregaten zeigten Aktivierungsmerkmale wie die starke Oberflächenexpression von CD69, einem frühen Marker, sowie CD25, das erst später hochreguliert wird. Dieser Befund konnte in vier verschiedenen Modellen bestätigt werden.

Cluster als 'funktionelle Einheiten' der T-Zellaktivierung

Experimente mit CFSE-markierten T-Zellen zeigten, daß 12-16 Stunden nach Transfer die ersten Lymphozyten in Clustern lokalisiert werden konnten und nach weiteren 18-20 Stunden ihre erste Teilung durchlaufen hatten. Diese APZ-abhängige Anfangsphase der Proliferation wurde später von einer vom Zell-Zell-Kontakt unabhängigen Phase abgelöst. Insgesamt benötigte die ‚T-Zellwelle' (auf Einzelzellebene konnte eine Analyse mit unserer Methode nicht durchgeführt werden) vom ersten Kontakt an ca. 32-36 Stunden, um das Clusterkompartment zu durchlaufen. Dieser Zeitrahmen war invariant und konnte in verschiedenen Modellen bestätigt werden, die sowohl beide T-Zelllinien (CD4, CD8) als auch zwei unterschiedliche MHC-Typen ($H2^b$,$H2^d$) umfassen. Darüber hinaus konnte beobachtet werden, daß T-Zellen, die für ein subdominantes Epitop und einen schwachen Agonisten spezifisch sind, eine gegenüber Lymphozyten, die ein dominantes Epitop erkennen, längere Zeitspanne brauchten, um die erste Zellteilung zu vollenden.

Das hCRP Modell: Cluster bieten eine Möglichkeit der Zellkommunikation

Eine wichtige Voraussetzung für die Kommunikation zwischen an einer adaptiven Immunantwort teilnehmenden Lymphozytenpopulationen ist die Präsentation der von ihnen erkannten Epitope auf ein und derselben APZ. Mit unserem Ansatz der Cluster-Isolierung und

dem Vorteil, im hCRP-Modell auf klonale T-Zellpopulationen mit Spezifität für zwei Epitope desselben Proteins zurückgreifen zu können, konnten wir zum ersten Mal zeigen, daß diese Konstellation *in vivo* existiert. Dies bot uns die Möglichkeit, den gegenseitigen Einfluß beider Populationen unter verschiedenen Bedingungen der Antigenverfügbarkeit zu untersuchen. In vorangegangenen Experimenten konnte gezeigt werden, daß sowohl bei Transfer nach Immunisierung als auch in antigenexprimierende Tiere die klonale Expansion von Zellen, die das dominante Epitop von hCRP erkennen, etwas höher war, als die der Zellen, die gegen das subdominante gerichtet waren. In beiden Fällen konnte kein gegenseitiger Einfluß nach Ko-Transfer beobachtet werden. Wurde das Antigen bei Immunisierung titriert, hatte dieses bis zu einer bestimmten Dosis bei beiden Populationen weder Einfluß auf den Zeitpunkt des Proliferationsbeginns noch auf deren Ausmaß. Wenn das Antigen intravenös appliziert wurde, also nur für sehr kurze Zeit verfügbar war, war die Antwort der gegen das dominante Epitop gerichteten Zellen in einer dosisabhängigen Weise wesentlich ausgeprägter als die der gegen das subdominante gerichteten Lymphozyten. Interessanterweise konnte bei Ko-Transfer unter diesen antigenlimitierenden Bedingungen eine deutlich erhöhte Expansion der für das subdominante Epitop spezifischen Zellen beobachtet werden.

Zusammenfassend sei angemerkt, daß die hier gezeigten Ergebnisse im Kontext der aktuellen Literatur eine Möglichkeit bieten, derzeitige Modelle der T-Zellaktivierung in einem neuen, erweiterten Modell zu vereinigen. Darüber hinaus stellt der Ansatz der Clusterisolierung eine Methode dar, die in Verbindung mit anderen experimentellen Ansätzen genutzt werden kann, um wichtige Fragen der frühen T-Zellaktivierung im Detail zu untersuchen.

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Abbreviations

aa	amino acids
AICD	activation induced cell death
Ag	antigen
APC	antigen presenting cell(s)
bp	base pairs
BrdU	bromodesoxyuridine
ccpm	Cerenkov counts per minute
CD	cluster of differentiation (cell surface markers)
CD25	IL-2 receptor β-chain
CD62L	L-selectin
CFA	complete Freund's adjuvant
Ci	Curie
conc.	concentrated
CRP	C-reactive protein
cTEC	cortical thymic epithelial cells
CTL	cytotoxic T-lymphocytes
dATP	desoxy-adenosine-triphosphate
dCTP	desoxy-cytidine-triphosphate
dGTP	desoxy-guanosine-triphosphate
dTTP	desoxy-thymidine-triphosphate
DC	dendritic cell(s)
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTPS	1:1:1:1 mixture of dATP, dCTP, dGTP, dTTP
EDTA	ethylene-diamine-tetra-acetate
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting/ scan
FCS	fetal calf serum
FDC	follicular dendritc cells
Fig.	figure
FITC	fluoresceine-isothiocyanate
Gy	Gray; unit of radioative dosis; 1 Gy = 100 rad
hCRP	human C-reactive protein
HEPES	4-(2-hydroxyethyl)1-piperazine-ethanesulfonic acid
HLA	human leukocyte antigen
^{3}H-Td	^{3}H-thymidine
ICAM	intercellular adhesion molecule-1
i.e.	that means
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
IS	immunological synapse
i.v.	intravenous
LC	Langerhans cell
LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
Mϕ	macrophage
mAb	monoclonal antibody
MACS	magnet-activated cell sorting
max.	maximum

MBP	myelin basic protein
mCRP	mouse C-reactive protein
MHC	major histocompatibility complex
min.	minimum
mSAP	mouse serum amyloid protein
mTEC	medullary thymic epithelial cells
MTOC	microtubuli organizing center
NK	natural killer cell
OD	optical density
o/n	over night
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
rpm	rounds per minute
RT	room temperature
s	seconds
SDS	sodium dodecylsulfate
SMAC	supramolecular activation cluster
SSC	sodium chloride/sodium citrate
TAE	tris/acetate/EDTA
TCR	T cell receptor
tg	transgenic
TGF-β	transforming growth factor-β
Th0	IL-2-secreting T effector cells
Th1	T helper 1
Th2	T helper 2
Tris	trihydroxy-methylamino-methane
TCR	T cell receptor
T_{reg}	T regulator cells
UV	ultra violet
VCAM	vascular cell adhesion molecule
VLA	very late antigen(s)
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

Declaration

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschliesslich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß sie abgesehen von unten angegebenen Teilpublikationen noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Jonathan C. Howard betreut worden.

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