Doctoral Thesis

Generation of TCR transgenic mice to study virus-specific CD4⁺ T cell responses in vivo

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Generation of TCR transgenic mice to study virus-specific CD4+ T cell responses in vivo

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Summary

Antigen-specific immunity on the level of CD4+ T cells has been mostly addressed using chemically defined, soluble protein antigens. Thanks to valuable conceptual knowledge emerging from these studies, it is now possible and of great interest to study CD4+ T cell functions in biologically relevant infectious disease models - especially since viruses may often behave differently as compared to soluble protein antigens in terms of antigen presentation, dynamics of antigen load and in their ability to activate multiple immune effector functions.

Thus, virus-specific CD4+ T cell responses induced by infection with non-cytopathic lymphocytic choriomeningitis virus (LCMV) were characterized focussing on several aspects: i) MHC class II-associated antigen presentation of LCMV-derived epitopes, ii) requirements for in vivo activation of LCMV-specific CD4+ T cells and iii) the generation and functional characterization of TCR transgenic mice with specificity for a LCMV-derived Th cell epitope.

Chapter 1 of the results section describes the molecular analysis of LCMV-derived Th cell epitopes and in vitro antigen presentation characteristics of these LCMV-derived MHC class II-binding epitopes upon infection of professional antigen presenting cells (APCs). Intracellularly synthesized, membrane-associated LCMV glycoprotein was found to be able to load MHC class II molecules independently of processing in acidic compartments and independently of the presence or absence of the invariant chain whereas intracellularly synthesized, cytosolic LCMV nucleoprotein was not able to load MHC class II molecules in LCMV infected APCs. This alternative MHC class II presentation pathway of endogenously synthesized LCMV glycoprotein was not only functional in vitro but also in vivo upon LCMV infection of invariant chain-deficient mice, as demonstrated in chapter 2 of the results section.

Chapter 3 analyzes the in vivo requirements of CD40-CD40L interaction either for the induction of humoral, virus-specific immune responses or, on the other hand, for the functional activation of virus-specific CD4+ T cells.

The fourth chapter of the results section describes the generation of TCR transgenic mice specific for an epitope of the LCMV glycoprotein. Phenotypical and functional characterization of these mice focussed on the antiviral protection potential of virus-specific CD4+ T cells as well as on the capacity of virus-specific Th cells to mediate help for antibody production against different virus-derived proteins.
Zusammenfassung

Antigenspezifische Immunität auf der Ebene von CD4+ T Zellen wurde hauptsächlich mittels chemisch definierten, löslichen Proteinantigenen untersucht. Da diese Untersuchungen zu wertvollem Grundlagenwissen führten, ist es nun möglich und von grossem Interesse, CD4+ T Zellfunktionen in biologisch relevanten infektiösen Modellsystemen zu evaluieren, insbesondere weil sich Viren und lösliche Proteinantigene oft unterschiedlich verhalten was Antigenpräsentation, Antigendynamik und die multiplen, immunaktivierenden Eigenschaften von Viren anbelangt.


Kapitel 3 des Resultateteiles analysiert die Notwendigkeit der CD40-CD40Ligand Interaktion einerseits für die Induktion von humoralen, virus-spezifischen Immunantworten und andererseits für die funktionelle Aktivierung von virus-spezifischen, CD4+ T zellen.

1. GENERAL INTRODUCTION

Every living organism is under continuous attack from its environment. To survive, they therefore have to develop defence mechanisms that render them resistant, or immune, to such attack. Even the most primitive organisms such as bacteria have developed simple defence mechanisms: their restriction enzymes can distinguish between bacterial and foreign DNA by different methylation patterns (Watson et al., 1987). Multicellular organisms have evolved more sophisticated defence mechanisms where coordinated interactions of different cell types are responsible for the elimination of pathogens. Two distinct branches of the immune system have evolved during evolution:

Innate immunity represents the original branch of the immune system which is evolutionary old and partly already present e.g. in the form of macrophages in primitive polyps. In vertebrates, innate immunity is present from birth on and is composed of many non antigen-specific factors such as physical barriers (skin and mucous membranes), phagocytic cells (polymorphonuclear leukocytes, macrophages) and biologically active substances (cytokines, degradative enzymes, toxic free radicals and growth inhibitors). In combination with the complement system, this non-specific immunity acts as a highly efficient primary defence against pathogens (Mims, 1982; Mims, 1987).

In contrast to innate immunity which is an attribute of almost every organism, acquired immunity has developed late in evolution and is only found in vertebrates. The key features of acquired immunity are its antigen-specificity and its ability to learn. The first encounter with a pathogen triggers a chain of events that induces a response with specificity against this pathogen and subsequently a status of specific memory is established: upon a second encounter with the same pathogen this antigen-specific memory allows a faster and more efficient secondary immune response.

The major cell types participating in acquired immunity are the T and B lymphocytes which carry highly variable antigen receptors allowing specific interactions with epitopes derived from pathogens or toxins. As initially proposed by Burnet in his clonal selection theory (Burnet, 1959), a given lymphocyte carries receptors of one specificity only enabling the selective involvement of antigen-specific lymphocytes in an immune response. The repertoire of specificities of lymphocytes is extremely broad with the potential to recognize most immunogenic determinants derived from the large pool of antigens and it is shaped such that lymphocytes carrying receptors for self determinants are specifically deleted (or silenced) and lymphocytes carrying receptors which can potentially interact with foreign determinants are positively selected.
There are two major components of acquired immunity: humoral immunity which is mediated by specific antibodies and cellular immunity which is mediated by different effector functions of antigen-specific T cells. B cells carry surface immunoglobulins which recognize antigens in their native form. Once a B cell is specifically activated it secretes a soluble form (antibody) of the membrane bound immunoglobulin. T cells, on the other hand, also carry membrane bound antigen receptors (T cell receptors, TCRs) which recognize antigen-fragments (derived from degraded protein antigens) in association with major histocompatibility antigens (MHC) (Bjorkman et al., 1987b; Maryanski et al., 1986; Townsend et al., 1985; Townsend et al., 1986; Zinkernagel and Doherty, 1974). According to their effector functions, to their expression of the CD4 or CD8 surface molecules and to their restriction to MHC class I or MHC class II molecules, T cells are divided into cytotoxic T cells (CD8+) and helper T cells (CD4+).

The mechanisms governing the above mentioned lymphocyte selection processes are well defined in the case of T cells but much less is known yet about B cell selection processes, i.e. whether or not they are at all positively selected amongst the primary B cell repertoire is not established yet.

Besides antigen-specific lymphocytes, macrophages and dendritic cells are crucially involved in the successful initiation of an immune response. Acting as professional antigen presenting cells (APCs) they process and present antigenic epitopes to T cells in an immunogenic fashion.

This introduction is divided into two major chapters: In a first chapter the features of the specific immune system will be described focusing on lymphocyte development, generation of lymphocyte diversity and lymphocyte selection processes. The second chapter concentrates on the generation of an antiviral immune response: on entry and spread of a virus, antigen presentation, activation of virus-specific T and B cells, effector functions of virus-specific lymphocytes and on induction of immunological memory.
A. Antigen specific immunity

T lymphocytes

Mature T cells have to fulfill certain requirements in order to effectively participate in an immune response: i) The specificity repertoire of T cells is initially generated randomly to maximize the possibilities of recognizing a vast number of structurally different epitopes. ii) T cells have to express surface TCR molecules which are able to recognize foreign antigenic fragments in association with self MHC molecules (MHC restriction) - thus they have to be positively selected for a certain degree of recognition of self-MHC molecules and, on the other hand, they have to be deleted or functionally inactivated if they are potentially autoreactive (i.e. if they recognize self epitopes in association with self MHC molecules). iii) T cells - originating from a common precursor cell - have to undergo a lineage commitment step either into the CD4+ or CD8+ T cell compartment. All three processes take place in the thymus and will be summarized in the following paragraphs.

A.1. T cell ontogeny, maturation and generation of diversity

In the mouse, lymphoid stem cells begin to appear in the thymus at day 11 of gestation. These cells enter the thymus from the bloodstream and presumably arise in the liver (hematopoiesis is prominent in the fetal liver and very limited in bone marrow until late in ontogeny). Whether thymic stem cells are precommitted to T cell differentiation is controversial. After entering the thymus, stem cells rapidly express high levels of Thy-1 molecules and proliferate extensively. At this timepoint the RAG enzymes (Brändle et al., 1992; Mombaerts et al., 1992; Oettinger et al., 1990) are expressed which leads to a rearrangement of the gene segments encoding the variable region of the TCR. A functional variable region of a TCR is composed of genomically juxtaposed gene fragments, two for the α-chain and three for the β-chain. The pool of gene segments involved in this random rearrangement consists of V-gene segments, D gene segments (for the β-chain) and J gene segments (Toyonaga and Mak, 1987). In a first step the β-chain rearranges V, D and J gene segments. If the recombination leads to a successful in frame rearrangement, the β-chain protein is expressed on the cell surface together with the recently described gp33 molecule (Groettrup and von, 1993). The expression of this complex prevents further rearrangement of the β-chain gene segments on the second chromosome (allelic exclusion) (Davis and Bjorkman, 1988; Marrack et al., 1987; Pircher et al., 1990b; Uematsu et al., 1988).
In a second step the α-chain gene segments are randomly rearranged from a pool of \(V\alpha\) and \(J\alpha\) gene segments. Apparently, the 'allelic exclusion' of the α-chain locus is not as stringent as the one of the β-chain locus since it has been demonstrated that approximately one third of the peripheral T cells express two different α-chains (Heath and Miller, 1993; Padovan et al., 1993). However, one of the two α-chains will most probably not contribute to a functional TCR in most cases since the stringent selection processes shaping the T cell repertoire will most probably only be met by one T cell receptor per cell.

The repertoire generated is further diversified by random N-region additions at the joining sites of the variable region gene segments (Bogue et al., 1992; Quertermous et al., 1986; Siu et al., 1984). The functional significance of this additional diversification remains obscure; although some influence on the fine-specificity of T cells could be demonstrated, anti-viral immune responses were not found to be impaired in the absence of N-region additions (Gavin and Bevan, 1995; Gilfillan et al., 1995).

Correlated with the expression of the rearranged TCRs on the thymocyte surface both the CD4 and CD8 accessory molecules are expressed (double positive thymocyte). At this developmental stage - in order to survive- the thymocyte has to undergo a critical selection process where the TCR has to meet distinct requirements in terms of avidity to self-MHC molecules expressed in thymic epithelium and the T cell has to undergo a lineage commitment step accompanied by downregulation of either CD4 or CD8 on the cell surface (single positive thymocyte). A lymphocyte fulfilling all these requirements will enter the peripheral T cell pool as a mature, naive T cell ready to be activated by antigen. These selection processes are discussed in the next chapter.

A2. T cell selection

The process of random genomic rearrangement generates a large number of structurally different TCR molecules from a rather limited amount of genetic information. A consequence of this random process will be (besides the generation of useful TCRs) the generation of TCR variable regions which are either useless (no recognition of self-MHC) or, on the other hand, dangerous (potentially self-reactive) for the individual. Thus, a strict selection process has to resolve these problems. Immature thymocytes expressing a functional TCR on their surface engage with self-MHC molecules expressed on thymic epithelium, on bone marrow-derived dendritic cell (DC) immigrants and on other APCs. If the TCR of an immature thymocyte interacts with self-MHC molecules (loaded with self peptides) on thymic epithelial cells with a minimal overall avidity of TCR-MHC interaction, the corresponding
thymocyte is rescued from programmed cell death by induced upregulation of bcl-2 (Benoist and Mathis, 1989; Blackman et al., 1986; Cory, 1995; Kisielow et al., 1988b; MacDonald et al., 1988a; Sha et al., 1990; VonBoehmer, 1986) (positive selection). This minimal avidity interaction selection process enables the T cell to potentially recognize self-MHC molecules loaded with foreign peptides with high avidity. If, on the other hand, the thymocyte does not interact with self-MHC molecules on thymic epithelial cells, i.e. when the overall avidity of TCR-self-MHC interaction is below a minimal threshold level, the thymocyte is not rescued from programmed cell death and will thus be eliminated from the T cell repertoire. Thus, 'useless' TCRs which are not interacting with self MHC molecules are deleted from the repertoire (Cohen et al., 1992). If the TCR-self-MHC interaction exceeds a critical threshold level, the thymocyte dies by activation induced cell death (Ashton-Rickhardt et al., 1994; Kappler et al., 1987; Kisielow et al., 1988a; Miller, 1989; Nossal, 1983; Pircher et al., 1989a; Sha et al., 1988) (negative selection). The negative selection of these high-avidity interacting thymocytes is a crucial step in the establishment of central tolerance where self-reactive T cells are physically deleted from the mature T cell repertoire. The positive selection is an active process with signal transduction via the TCR-CD3 complex leading to upregulation of surface activation markers such as CD69 (Brändle et al., 1994; Swat et al., 1993), expression of 'survival' molecules such as bcl-2 (Strasser et al., 1991) and a shutdown in RAG-enzyme synthesis thereby terminating TCR rearrangement (Brändle et al., 1992). Thus, two different threshold levels of the overall avidity of TCR-MHC interaction apparently govern thymic selection processes: A minimal threshold level allows positive selection whereas a maximal threshold level leads to negative selection. Interestingly, intracellular signaling pathways mediating positive selection can be separated from signals mediating negative selection, since the ras-signaling pathway is apparently essential for positive but not for negative selection (Alberola-Ila et al., 1996).

So far the T cell has fulfilled the criteria of positive and negative selection and in a further selection step the thymocyte has to undergo a lineage commitment either to the CD4+ T helper cell compartment or to the CD8+ cytotoxic T cell compartment. Two different theories have been suggested to govern this lineage commitment process: The stochastic model of lineage commitment claims that a double positive thymocyte randomly downregulates the expression and synthesis of either CD4 or CD8 (Robey and Fowlkes, 1994). Engagement of the TCR with MHC molecules on thymic epithelial cells will divide the thymocytes into MHC class I- and MHC class II-interacting cells depending on the structural characteristics of the TCR. If such a cell has, by chance, the appropriate coreceptor on the surface matching the recognized MHC molecule, it will be positively driven into the respective compartment, whereas
a thymocyte expressing the wrong coreceptor will be deleted (Chan et al., 1993; Davis et al., 1993; Itano et al., 1994; van Meerwijk and Germain, 1993).

The instructive model of lineage commitment proposes that a double positive thymocyte engages its TCR with self-MHC molecules and according to the MHC molecule recognized either CD4 or CD8 is involved in MHC binding. This will be followed by instructive downregulation of the wrong coreceptor (Borgulya et al., 1991; Robey et al., 1991). There are still controversial discussions favouring one or the other model and, in addition, some reports suggest, that the lineage commitment processes may be not as clearly dissectable in one or the other mechanism but rather that different pathways govern CD4+ T cell and CD8+ T cell commitment. In particular, it has been suggested that CD4+ lineage commitment is a CD4-independent default pathway while CD8+ lineage commitment requires signals delivered by the CD8 coreceptor (see appendix ((Bachmann et al., 1995a))(Lucas et al., 1995; Lundberg et al., 1995; Suzuki et al., 1995)).

A central role of thymic selection processes is to ensure the development and maturation of MHC-restricted T cells capable of potentially recognizing foreign antigenic structures as well as the selective deletion of self-reactive T cells. The process of negative selection in the thymus can only occur for self-antigens which are either expressed in the thymus, transported to the thymus in sufficient amounts or - at least theoretically - for self-antigens which are derived from the extremely high number of thymocytes undergoing apoptosis in the thymus. Many studies have shown the induction of central tolerance in antigen-specific model systems using TCR-transgenic mice (Blüthmann et al., 1988; Kisielow et al., 1988a; Pircher et al., 1989a; Sha et al., 1988) or endogenously expressed superantigens (Kappler et al., 1987; Kappler et al., 1988; MacDonald et al., 1988b; MacDonald et al., 1988c; Pircher et al., 1989b).

Several groups have reported that tolerance induction in the thymus can also occur by non-deletional clonal inactivation (anergy) rather than by physical deletion (Ramsdell et al., 1989; Roberts et al., 1990). In these cases tolerance induction occurs at a very late stage of thymic maturation or even in the periphery (Blackman et al., 1990).

In the case of self antigens which are expressed exclusively in the periphery, self-specific T cells will not be deleted in the thymus and will possibly encounter self-antigen in the periphery. At least three different mechanisms/fates of self-reactive T cells have been experimentally documented: T cell deletion (physical elimination) (Bertolino et al., 1995), T cell anergy (functional inactivation) and T cell ignorance (self-reactive T cells do not 'see' the antigen and do not change their reactivity).

Induction of T cell anergy has initially been performed in vitro by inappropriate stimulation of T cell clones (Schwartz, 1990). According to the 2 signal theory
initially proposed by Bretscher and Cohn for lymphocytes in general (Bretscher and Cohn, 1970), signal 1 is delivered by TCR-MHC engagement and signal 2 is a costimulatory signal, either a cell associated interaction of additional receptor-ligand pair(s) or soluble mediators (Armitage et al., 1992; Harding et al., 1992; Linsley et al., 1991b; Mueller et al., 1989; Schwartz, 1990). Signal 1 alone induces a state of anergy in the T cell which may be reversed into a responsive state if an appropriate signal 1 and 2 is provided (Schwartz, 1990). In vivo, anergic T cells have been characterized with the availability of TCR transgenic mouse models offering the opportunity to physically observe self-reactive T cells in the periphery. In fact, depending on the geographical location of the peripherally expressed (neo) self-antigen, on the expression level of the (neo) self-antigen and on the cell type expressing the (neo) self-antigen different stages of T cell unresponsiveness have been described (Arnold et al., 1993; Ferber et al., 1994; Hämmerling et al., 1993; Hämmerling et al., 1991; Schönrich et al., 1991; Schönrich et al., 1992).

Ignorance of self-reactive T cells towards a self-antigen is observed if the self-antigen is not accessible for naive T cells, i.e. if the self-antigen is sequestered in tissues usually not under surveillance by naive T cells (e.g. CNS, pancreas) (Barker and Billingham, 1972). This has been described for example for a neo-self-antigen of viral origin expressed in the β-cells of the pancreas (Oehen et al., 1994; Ohashi et al., 1991; Oldstone et al., 1991).

Nevertheless, both situations (anergy and ignorance) might be dangerous under certain circumstances where i) anergic T cells in certain stages of unresponsiveness might be 'rescued' if the appropriate second signal is provided e.g. by interleukin levels after an infection (Kawai and Ohashi, 1995) or ii) if tissue injury/trauma leads to transport of the sequestered self-antigen into secondary lymphoid organs where they will be presented by professional APCs capable of activating naive or anergic T cells (Sinha et al., 1990) or iii) if self-reactive T cells are activated by an unrelated crossreactive foreign antigen.

Peripheral tolerance induction can be induced experimentally by different approaches: Peripheral tolerance induction by means of deletion of antigen-specific T cells has been demonstrated after overwhelming viral infections leading to T cell exhaustion (Moskophidis et al., 1993). Exhaustive activation/elimination of virus-specific T cells was observed after infection with high doses of widely replicating non-cytopathic virus finally inducing a virus-carrier status. This exhaustive activation/elimination of virus specific T cells might be viewed as self-protective mechanism for the host against deleterious immunopathology: if virus replication is outstripping the immune response it might be advantageous for the host to specifically inactivate the virus-specific immune response and to tolerate the virus infection - if it is a noncytopathic virus - instead of causing lethal immunopathology. Thus, antigenic burden and kinetic
balances of viral replication and T cell induction play a crucial role in this exhaustive T cell activation/deletion mechanism.

Similarly, mouse mammary tumour virus (MMTV) infections have been shown to specifically delete superantigen-reactive Vβ -T cell populations (Acha et al., 1993). Peripheral tolerance induction with non-replicating antigens by means of inactivation/deletion has also been achieved using a defined regimen of antigen-administration as shown for immunogenic peptides (Aichele et al., 1994; Briner et al., 1993; Gaur et al., 1992; Ria et al., 1990; Smilek et al., 1991) and for immunogenic proteins in the case of CD4+ T cells (Chiller et al., 1971; Dresser, 1962; Mitchison, 1964; Weigle, 1961).

B lymphocytes

Mature B cells must - in analogy to the T cells - be able to recognize a wealth of different structural determinants, although the structural requirements demanded from immunoglobulins are not as stringent as for the TCR. As described previously, the TCR has to fulfill the criterion of MHC restriction, i.e. it has to be able to differentiate between self-MHC+self-peptide and self-MHC+foreign peptide. For the immunoglobulin it has not been shown that it has to be restricted to a self-component. Thus, the generation of antigen-binding sites of immunoglobulins and the maturation of a B cell is theoretically only limited by successful rearrangement of the gene fragments composing the variable regions and by successful pairing of the heavy and light chains - unless, because of random generation of the antigen-binding sites - it is specific for a self-antigen (see below).

A 3. B cell ontogeny, maturation and generation of diversity

The bone marrow is the main site of B cell ontogeny and maturation in adult mammals. During the development of B cells from bone marrow-derived stem cells to mature naive B cells expressing IgM and IgD immunoglobulins, several stages of B cell development have been defined according to the expression of different surface markers: B cells develop from pro B cells to pre B cells to immature B cells and to mature B cells (Melchers et al., 1989).

In the stem cell precursor the genes encoding the genetic components of the variable region of an immunoglobulin (V-gene segments, D-gene segments in the case of the heavy chain and J-segments) are still in germline configuration. During B cell ontogeny the heavy chain variable region gene segments (VH, D, JH) are rearranged
before those of the light chain (\(V_L, J_L\)) in analogy to the TCR V-region where rearrangements in the \(\beta\)-chain locus preceede those on the \(\alpha\)-chain locus (Alt et al., 1982; Tonegawa, 1983). RAG enzyme activities are also a prerequisite of B cell genomic rearrangement (Oettinger et al., 1990; Schatz et al., 1989). In addition, the terminal desoxyribonucleotide transferase (TdT) randomly introduces nucleotides (N-nucleotides) at the sites of segment joining (Desiderio et al., 1984). The successfully rearranged heavy chain gene is expressed intracellularly in pre B cells, associates with the surrogate light chain (Karasuyama et al., 1994; Melchers et al., 1994) and inhibits further rearrangement of \(V_H\) gene segments on the second chromosome leading to allelic exclusion (Weaver et al., 1985). These mechanisms closely resemble the ones observed in T cell maturation where association of rearranged \(V\beta\)-chains with gp33 inhibits further rearrangement of \(\beta\)-chain genes. As shown for T cells, the allelic exclusion is less stringent for the light chain as for the heavy chain (Giachino et al., 1995).

In a next step the light chain gene segments (\(V_L\) and \(J_L\)) undergo genomic rearrangement, are expressed and associate with the heavy chain leading to the expression of a surface IgM molecule. Finally B cells express in addition to the IgM isotype the IgD isotype of a given antigen binding site and thus become mature B cells (Paul, 1989).

**A 4. B cell selection**

It has been known for a long time that tolerance induction on the B cell level is not as tightly regulated as on the T cell level since self-reactive B cells are found in measurable frequencies in the periphery of adult mice (Weigle, 1980). B cell activation is usually dependent on Th cells, thus it could be assumed that self-reactive B cells would remain unactivated as long as Th cell tolerance was established (Mitchison, 1964; Möller, 1975). The first model allowing the study of B cell tolerance in vivo at the clonal level was a double-transgenic mouse where an immunoglobulin specific for hen egg lysozyme (HEL) was coexpressed with a soluble form of HEL (Goodnow et al., 1988). This model clearly demonstrated a nondeletional form of B cell inactivation similar to the anti-hapten clonal anergy model that was described by Nossal and Pike in normal mice (Nossal and Pike, 1980). In the double transgenic mice HEL-Ig bearing B cells were present at the same frequency as in HEL-Ig single transgenic mice. However, these B cells exhibited a strongly reduced expression of surface IgM and were shown to be functionally inactive (Goodnow et al., 1989). As shown for unresponsive T cells this nonresponsive status was reversible - either by activation with LPS or by transfer of
the nonresponsive B cells into HEL-free recipients (Goodnow et al., 1991). The nonresponsive B cells in the double transgenic mice were shown to have a drastically reduced life span of 3-4 days as compared to the lifespan of 4-5 weeks in the anti-HEL-Ig single transgenic mice (Fulcher and Basten, 1994). Thus, albeit this mechanism for purging the peripheral B cell repertoire of self-reactive B cells is not as rapid as has been observed transgenic mice expressing a membrane-bound form of HEL (Hartley et al., 1993), it nevertheless contributes to the elimination of self-reactive B cells in the primary B cell repertoire. This suggests that anergy and deletion are not totally dissimilar mechanisms but rather may form part of a spectrum. Both B cell deletion and anergy are usually induced in immature B cells in the bone marrow (Hartley et al., 1991; Nemazee and Bürki, 1989a; Nemazee and Bürki, 1989b) but can also occur in the periphery with mature B cells (Goodnow et al., 1989; Russell et al., 1991). This may - in analogy to anergy induction in T cells - be another example strengthening the 2 signal theory of lymphocyte activation: mature B cells bind antigen via surface Ig (signal 1) in the absence of signal 2 because T help is tolerant and because no inflammation is occurring which could substitute for cognate T help and thus signal 1 alone would lead to deletion or induction of non-responsiveness of self-reactive B cells.

The fate of self-reactive B cells (i.e anergy versus deletion) seems to depend on the overall cross-linking ability of the self-antigen: the more cross-linking the antigen for the surface Ig molecules, the deeper the tolerance induced (anergy (little crosslinking) - deletion (much crosslinking). As shown by Bachmann et al., the same criteria seem to govern the immunogenicity of a B cell antigen: the more crosslinking the B cell antigen the more immunogenic its character (Bachmann et al., 1995).

Another aspect to be considered is tolerance within the secondary B cell repertoire: During the process of hypermutation of immunoglobulin variable region genes in germinal centers, a secondary B cell repertoire is generated with specificities potentially different from the primary B cell repertoire. Given the high frequency of variable gene mutations, it is unavoidable that from time to time a B cell by chance acquires self-reactivity. Several fates of such a cell could be envisaged: i) it might not be positively selected by the special antigen-capturing/selection mechanism of the germinal center (Nossal and Ada, 1971a) and would thus die or ii) it would be positively selected by self-antigen present in the germinal center but would not be further activated since self-antigen specific T help is tolerant (T cell tolerance is induced at lower antigen concentrations than B cell tolerance) (Mitchison, 1968; Zinkernagel et al., 1991) or iii) it would interact with the self antigen and die by apotosis (Cornall et al., 1995; Jacob and Kelsoe, 1992; Pulendran et al., 1995).
B. Antiviral immune response

Comparison of viruses with non-replicating antigens

In contrast to non-replicating antigens, viruses (and their antigenic determinants) multiply and thereby exhibit complex kinetics. Viruses and the immune system are directly interacting partners; various effector functions of the immune system (cytotoxic T cells, soluble factors, antibodies) interfere with viral replication and host cell infection aiming to clear the virus from the individual whereas viruses may escape immunosurveillance by various means (due to latency, due to mutations in immuno-relevant epitopes, due to sequestration in geographical sites not accessible by the immune system or due to silent integration of viral DNA into the host genome (retroviruses)).

Viral antigenic B cell determinants may be classified into two subsets: protective immunological target antigens and non-protective immunological target antigens. Protective target antigens are viral epitopes recognized by neutralizing antibodies and are expressed on the viral surface as opposed to non-protective target antigens which are virus-internal proteins which rather have properties of common immunological model antigens such as ovalbumin, hen egg lysozyme and others.

Viral infections efficiently trigger a series of events including host cell lysis (either by the virus itself or by immunopathology), inflammation and cytokine production. In contrast, non-replicating antigens do not induce these responses and therefore usually require adjuvants for the induction of local inflammation and/or cytokine induction.

B 1. Viral characteristics, entry and spread within the host

Higher vertebrates are infected by viruses via skin, mucosal surfaces and some viruses may enter the blood directly (e.g. via insect bites). The mandatory infection of a host cell is usually targeted by the interaction of viral surface molecules with receptors on host cells (e.g. MHC class I molecules for Semliki forest virus and Simian virus 40, growth factor receptors for Vaccinia virus, beta-2-microglobulin for Cytomegalovirus, I-CAM for rhinoviruses etc.) (Zinkernagel, 1993). After initial local replication has taken place, most viruses reach local lymph nodes (LN) either directly or via transport by professional APCs where they may proliferate further and eventually spread systemically thereby reaching secondary organs. Cell tropism, replication kinetics, extent of host cell destruction in the case of cytopathic viruses and various other parameters will vary from one virus to the other. Virus entry, spread
and tropism will directly determine the pathology caused by cytopathic viruses - or indirectly the immunopathology in the case of non-cytopathic viruses. Another variable parameter to be considered in the case of RNA viruses is their genetic instability due to the high error rate of their RNA-polymerase.

All viruses - being a foreign structure entering an organism - are usually noticed by the immune system, either on the surface of infected cells, as freely circulating viral particles or - most importantly - on antigen presenting cells which either take up and process viral antigens or are infected themselves. The antigenicity and immunogenicity of a virus depends on different parameters: i) The tropism of a virus determines if virally-derived determinants are efficiently presented to the immune system. Viruses initially infecting neuronal cells (e.g. rabies or vesicular stomatitis virus) (Fields, 1990) or epidermal cells (e.g. papilloma viruses) (Ferenczz et al., 1985) may be antigenic but not immunogenic since they avoid professional APCs. ii) Viral replication kinetics determine the antigenic burden presented to the immune system. iii) CTL-mediated cell lysis leads to release of viral particles and viral protein antigens increasing the amount of antigen accessible to T and B cells. The same process may, however, be deleterious if virus has already spread extensively. iv) Retroviruses integrated into the host genome are neither antigenic nor immunogenic since (almost) no viral proteins are expressed within the silently infected cell (Ada et al., 1992; Sabin, 1992). v) Some viruses - in particular DNA viruses - elaborated special strategies during evolution escaping the recognition by the immune system (e.g. Herpes simplex virus and murine Cytomegalovirus specifically retain MHC class I molecules in the ER, Epstein-Barr virus inhibits the processing of MHC class I epitopes, Adenovirus inhibits transcription activators for MHC class I synthesis (Spriggs, 1996)).

B 2. Antigen presentation

As is the case for most model antigens studied, viral antigens must be presented by professional APCs within the microenvironment of secondary lymphoid organs (Kündig et al., 1995) to efficiently induce an immune response. Viral antigens are transported to secondary lymphoid organs by different means: i) At the site of infection viral antigens may be released and taken up by local APCs (e.g. Langerhans cells in the skin representing dendritic cell (DC) precursors) which migrate subsequently to the local draining lymph node. The Langerhans cells differentiate there into mature DCs which are believed to be the most potent activators of naive T cells (Sallusto and Lanzavecchia, 1994). ii) Alternatively, viral particles or viral antigens may directly enter the spleen or LN via the blood or the lymph where they are filtered
out of the circulation by marginal zone macrophages or macrophages located in the subcapsular sinus (Germain and Margulies, 1993; Nossal and Ada, 1971b). The activation of naive lymphocytes exhibiting the appropriate specificities occurs within the T-regions of the LN or the spleen (Davies et al., 1969).

**MHC class I associated antigen presentation**

In general, MHC class I molecules present protein fragments (peptide epitopes) derived from intracellularly synthesized proteins. Thus, any MHC class I positive cell infected by a virus will normally synthesize viral proteins some of which are readily degraded within the cytosol by the proteolytic activity of the proteasome complex (Ciechanover, 1994; Monaco, 1992; Rock et al., 1994). Some of the generated peptides are transported from the cytosol into the ER via the TAP transporter molecules (Hill and Ploegh, 1995). The peptides entering the ER - if exhibiting appropriate MHC class I binding anchor residues (Falk et al., 1991) - will bind to newly synthesized MHC class I molecules associated with β2-microglobulin (β2-M) (Saper et al., 1991). The trimeric complex MHC class I / β2-M / peptide is subsequently transported to the cell surface by the normal secretory pathway (Braciale, 1992; Neefjes and Ploegh, 1988). On the cell surface the complexes can be recognized by specific CD8+ T cells.

Since almost all nucleated cells express MHC class I molecules, virtually any cell which is infected with a virus can express MHC class I molecules loaded with virus-derived peptide epitopes and thus be recognized by the immune system. This is of crucial importance for an efficient immunosurveillance since most cell types within an organism are potentially infectable by some viruses and therefore there should be no limitation to signal the immune system its infected status. It has to be emphasized, however, that, under normal circumstances, T cell priming is restricted to professional APCs and usually does not occur outside secondary lymphoid organs (see below).

Although the above described pathway may be the most efficient and the normal pathway for loading MHC class I molecules, some exceptions have been described in vitro and in vivo (Jondal et al., 1996; Schirmbeck et al., 1992; Staerz et al., 1987). In vitro MHC class I loading could be achieved using protein-coated beads (Rock et al., 1990), proteins associated with cellular debris (see appendix, (Bachmann et al., 1995b; Bevan, 1987)) or bacterial antigens (Pfeifer et al., 1993). In vivo cross-priming experiments with allogeneic cells carrying minor histocompatibility antigen differences (Bevan, 1976) or H-Y differences (Gordon et al., 1976) demonstrated induction of CD8+ T cells. In addition, CD8+ T cell priming was possible by immunization with virally-derived protein antigens (Bachmann et al., 1994a; Weidt et
MHC class II associated antigen presentation

MHC class II molecules are only expressed on a limited number of cell types such as professional APCs (DC and macrophages) and B cells. This concentrates MHC class II associated antigen presentation mostly to secondary lymphoid organs and to some APCs residing outside secondary lymphoid organs.

Antigen fragments presented on MHC class II molecules are generally derived from soluble, exogenous proteins/protein complexes which are taken up by APCs via different mechanisms: i) phagocytosis of protein complexes, ii) macropinocytosis of soluble antigens (as described for immature DC) (Sallusto and Lanzavecchia, 1994), iii) Fc- or complement-receptor mediated endocytosis of antigen-antibody complexes, iv) specific receptor-mediated uptake of soluble antigens, antigen complexes or viral particles by antigen-specific B cells. The fate of the internalized antigen is the same in all cases: The antigen is trapped in endosomes and successively transported from early to late endosomes, to the MIIC compartment and finally to lysosomes. During this transport process the environment becomes gradually more and more acidic allowing antigen degradation by acidic proteases which continuously degrade the antigen into peptidic fragments (Bevan, 1987; Germain, 1986; Morrison et al., 1986).

Newly synthesized MHC class II α and β chains associate with the invariant chain (Ii) within the ER. Several functions have been attributed to Ii: i) structural stabilization of the MHC class II αβ dimer (Cresswell, 1992), ii) physical inhibition of peptide binding to the MHC class II peptide binding groove and iii) targeting of MHC class II molecules to endosomal compartments (Guagliardi et al., 1990; Lamb et al., 1991; Neefjes et al., 1990) (Bakke and Dobberstein, 1990) and especially to the recently described MIIC compartment (Amigorena et al., 1994; Tulp et al., 1994). Having reached the endosomal compartments, Ii is subjected to proteolysis by acidic proteases and in particular, as recently described, by cathepsin S (Riese et al., 1996).

The remainder of Ii after proteolysis consists of a residual 20-24 mer fragment called CLIP (class II associated invariant chain peptide) which occupies the peptide binding groove of the MHC class II molecules (Ghosh et al., 1995). Within the endosomal compartments and mainly within the MIIC compartment, the residual CLIP peptide is exchanged with appropriate peptides derived from proteolysis of endocytosed antigens. This exchange reaction is catalyzed by the MHC class II gene cluster-
encoded H2-M molecule (Denzin and Cresswell, 1995; Miyazaki et al., 1996). Peptide loaded MHC class II molecules are finally transported to the cell surface where they can be recognized by CD4+ T cells. Similar to the exceptions mentioned for the MHC class I loading pathway, there have been described several exceptions to the 'classical' MHC class II loading pathway (Brooks et al., 1991; Dodi et al., 1994; Jacobson et al., 1989; Jaraquemada et al., 1990; Jin et al., 1988; Malnati et al., 1992; Nuchtern et al., 1990; Oxenius et al., 1995; Sekaly et al., 1988). In general, it has been observed that certain endogenously synthesized proteins (especially proteins that naturally reside in or pass through the ER during their biosynthesis) are able to load MHC class II molecules outside endosomal compartments (Weiss and Bogen, 1991). This may be of particular interest in the case of infections of professional APCs by non-cytopathic viruses, enabling MHC class II presentation of internally synthesized protein determinants before CTL-mediated destruction of infected cells liberates viral proteins for 'classical' antigen uptake. Chapter 1 of the results section will focus on a more detailed in vitro analysis of these 'non-classical' MHC class II loading pathways, while chapter 2 will analyze the consequences of this pathway during a viral infection in vivo.

**Antigens recognized by B and T cells**

B cells recognize antigens in their native form, therefore they do not require antigen processing as in the case of T cell antigens. Nevertheless, there are qualitative differences between B cell antigens with respect to their ability to activate B cells by binding to the surface Ig receptors. In general, the more repetitive and structurally organized a B cell antigen (e.g. proteins on the surface of a viral particle) the more potent is its ability to cross-link the B cell receptors and the more efficient is the B cell activation (Bachmann et al., 1995). In addition, the intrinsic B cell repertoire may be more or less adequately shaped for the recognition of certain B cell antigens (protecton, (Cohn and Langman, 1990)) as well as the accessibility of B cell epitopes on the surface of viral particles may determine the quality of a B cell antigen. Similarly, antigens differ in their quality to serve as T cell antigens: the quantity of the antigen may influence the efficiency of MHC molecule loading. In addition, the processing machineries generating MHC class I and II epitopes have to successfully degrade antigens, MHC molecule-demanded anchor positions and peptide lengths have to be fulfilled, cross-reactivities to self-epitopes have to be considered and a specific T cell precursor repertoire has to be existent for a certain antigenic structure. The most important feature determining the quality of a T cell epitope is the APC presenting the T cell epitopes which - in analogy to the structurally ordered B cell
antigens on the surface of a viral particle introduces a certain degree of order and repetitiveness in the T cell epitope by presenting the same relevant epitope on identical MHC molecules in multiple copies on the cell surface. In fact, T cells recognizing processed antigens rather than native antigens has two advantages for the immune system: i) it focusses the T cell on the antigen-presenting or target cell and ii) it literally turns the pathogen inside out: while protective B cell responses are limited to few structures on the surface of a pathogen which may easily be mutated and thereby escape immuno-surveillance, T cells recognize any adequate epitope of pathogen-derived proteins.

B 3. Lymphocyte activation

Naive mature lymphocytes enter the peripheral T or B cell pool emerging from the thymus or the bone marrow. Antigen-specific activation of these cells usually occurs in secondary lymphoid organs where antigens are presented within an optimal microenvironment. For efficient presentation of T cell antigens, secondary lymphoid organs harbor specialized antigen presenting cells capable of activating naive T cells. In the case of viral infections this is illustrated by the finding that most viruses capable of inducing an early and efficient CD8+ T cell response tend to infect professional APCs (e.g. LCMV, EBV (Zinkernagel, 1993)). The lymphocyte activation processes are complex and depend on different receptor-ligand interactions, cell-cell interactions and secretion of soluble mediators.

Since B cell activation is usually intimately connected to Th cell activation and to Th cell effector functions, the activation requirements for B cells will be discussed in the chapter T cell effector functions.

Activation of T cells

As initially proposed by Bretscher and Cohn for B cells (Bretscher and Cohn, 1970) and later for T lymphocytes (Lafferty et al., 1983; Schwartz, 1990), current dogmas evisage T cell induction as a two signal event. Signal 1 is antigen-specific and mediated by the interaction of the TCR with MHC and peptide; signal 2 is not antigen-specific although closely linked to the cognate interaction of APC and T cells. Initially this second signal was believed to represent soluble mediators (antibodies in the Bretscher and Cohn model and interleukins in the Schwarz model), whereas later studies revealed the importance of costimulatory ligand-receptor interactions between APC and T cell. In this respect, the interaction of CD28 on the T cell and B7.1 or
B7.2 on the APC has been shown to be crucial for efficient T cell activation (Armitage et al., 1992; Harding et al., 1992; Linsley et al., 1991a; Mueller et al., 1989; Schwartz, 1990). More recently the interaction between CD40 (expressed on B cells, macrophages, DC) and CD40L on Th cells has been described to play a major role in Th cell induction (Grewal et al., 1995; van Essen et al., 1995). Since CD40L is solely expressed on activated Th cells, this interaction can only become important after an initial activation step of the Th cell which obviously has to be CD40/CD40L independent.

The overall result of such a two-signal interaction of naive T cells with MHC-peptide complexes on APCs is the activation of a naive T cell governed by a complex cascade of biochemical intracellular signal transduction events, which take their origin at the TCR-associated CD3 complex and finally lead to transcriptional activation of a variety of genes in the nucleus (Weiss and Littman, 1994). The activated T cell starts to proliferate (clonal expansion) and to acquire T cell effector functions. For CD4+ T cells, effector functions involve the ability to secrete a certain pattern of cytokines and to upregulate CD40L which is crucial for T-B cooperation. CD8+ effector T cells gain the ability to lyse target cells expressing the appropriate MHC-peptide complex via perforin and/or fas-mediated cytolysis pathways and to secrete cytokines. A more detailed description of T cell effector functions will be given in the next chapter.

**CD4+ T cell activation**

Qualitative differences exist in the ability of antigen presenting cells to activate naive Th cells; DCs are the most potent activators of naive Th cells. Mature DCs have been shown to be poorly phagocytosing as opposed to immature DCs which are extremely efficient in trapping antigen via macropinocytosis (e.g. the Langerhans cells in the skin representing an immature DC as opposed to interdigitating DCs in secondary lymphoid organs). It is thus plausible that immature DCs in peripheral organs - e.g. at the site of infection - efficiently capture antigen and process it. In addition locally present inflammatory factors such as IL-1, TNFα or LPS induce the maturation of immature DCs to become fully competent T cell activators expressing high levels of MHC class II, of B7.1 and B7.2 (Sallusto and Lanzavecchia, 1994). This may in fact 'freeze' the antigenic status of the cell, preventing a rapid replacement of antigenic fragments after migration to lymphoid organs.

Macrophages are the main cell type able to phagocytose particulate antigens and might be expected to present such antigens to naive T cells (Unanue, 1984). Soluble antigens on the other hand might be expected to primarily be taken up by specific B cells which are able - by means of their surface Ig-receptors - to trap
locally and concentrate the soluble antigen and process and present antigenic fragments after receptor-mediated uptake. It is still a matter of debate whether B cells can function as APCs in inducing naive T cells, since resting B cells express low levels of MHC class II molecules and B7.2 and no B7.1 unless previously activated (Freeman et al., 1993; Gilbert and Weigle, 1994; June et al., 1994). While some investigations claim B cells to be tolerogenic for naive T cells (Eynon and Parker, 1992; Fuchs and Matzinger, 1992; Gilbert and Weigle, 1994; Ronchese and Hausmann, 1993) due to this lack of costimulation, other experimental conditions using soluble protein antigens find B cells to be instrumental in priming naive Th cells (Constant et al., 1995c; Janeway et al., 1987; Kurt-Jones et al., 1988; Liu et al., 1995; Rock et al., 1984; Ron and Sprent, 1987; Schultz et al., 1990) as well as for the expansion of antigen-primed T cells (Grewal et al., 1995; Linsley et al., 1991a; Singer and Hodes, 1983).

In the case of a viral infection which usually induces inflammatory processes and the secretion of soluble mediators (such as interferons, IL-1, TNFα and others), APCs may generally be more readily activated and thus may express higher levels of MHC and costimulatory molecules than after immunization with soluble protein. They therefore serve as more potent T cell activators as compared to APCs after immunization with a soluble protein antigen. In addition - as opposed to soluble experimental antigens - viral proteins are mostly not present in truly soluble form but rather as viral particles or associated with cell debris after cell lysis. Thus, phagocytosis by macrophages of viral antigens is probably favoured over B cell-mediated Ig receptor-linked uptake. In addition, viral replication leads to antigen concentrations increasing over time which differs from the administration of soluble proteins.

In line with the above mentioned CD40-CD40L interaction shown to be required for Th cell activation after immunization with soluble protein antigens (Grewal et al., 1995; van Essen et al., 1995), this interaction seems to be less critical for the induction of virus-specific Th cells as will be pointed out in chapter 3 of the results section. Thus, Th cell activation may be governed by slightly differing pathways after immunization with soluble antigens versus live viruses and most probably more subtle pathways are required for proteins to induce T cell activation.

An additional feature of Th cell activation becoming more and more a center of interest is the selective induction of Th cells capable of producing different patterns of cytokines. According to these cytokine production patterns, Th cells have been classified in Th1 (predominantly secreting IL-2, IFNγ and TNFα) and Th2 cells (predominantly producing IL-4, IL-5, IL-6 and IL-10). Th0 cells which do not exhibit such a restricted cytokine secretion pattern are believed to represent the precursor population of Th1 and Th2 cells. Although these discrete Th cell populations have
been clearly demonstrated amongst murine Th cell clones in vitro (Mosmann et al., 1986; O'Garra et al., 1993; O'Garra and Murphy, 1994; Swain et al., 1991; Swain et al., 1990), the distinction between such discrete populations proved to be more difficult in human Th cell clones and in in vivo investigations.

Upon T cell activation, various parameters may influence the development of Th cells exhibiting a certain cytokine secretion pattern. Initially, distinct Th cells phenotypes were experimentally achieved by culturing Th cell clones in vitro under certain cell culture conditions: addition of IFNγ and anti-IL-4 leading to Th1 development or addition of IL-4 leading to Th2 development. More recently such discrete Th cell populations have also been characterized in vivo on the effector cell level after infection with certain parasites which were shown to influence critically the control of the infection (Campbell et al., 1996; Kamanaka et al., 1996; Soong et al., 1996).

The parameters influencing the induction of a Th1-like or Th2-like Th cell response in vivo are still a matter of debate: i) antigen concentration (Hosken et al., 1995), ii) APC populations involved in Th cell priming (Duncan and Swain, 1994), iii) quality of the antigens (i.e. pathogens versus proteins versus peptides) (Constant et al., 1995a) iv) microenvironment at the Th cell priming site (e.g. presence of certain soluble mediators), v) genetic background of the animal (Hsieh et al., 1995) and vi) presence of CD40-CD40L interaction (Campbell et al., 1996; Kamanaka et al., 1996; Magram et al., 1996; Soong et al., 1996) have all been shown to be involved in directing Th cell responses in one or the other direction.

In the case of Leishmania infection, Th1 responses are observed in C57BL/6 mice whereas Th2 responses are observed in BALB/c mice. Viral infections usually induce a Th1 cytokine pattern in virus-specific Th cells whereas helminths or allergens promote Th2 development (Mosmann and Coffman, 1989a; Mosmann and Coffmann, 1989).

Th1 cells are thought to be involved in cellular immune responses such as delayed type hypersensitivity reactions (DTH), macrophage activation and activation of cytotoxic T cells and NK cells. Th2 cells on the other hand seem to be important in the induction of humoral immune responses, in allergic reactions and the elimination of helminths. Whether the importance of Th2 cells in inducing humoral responses holds true is questionable since it has also been shown that switching to different isotypes in B cells is selectively induced by Th1 or Th2 cells (Th1 cells promoting IgG2a responses (especially after viral infections); Th2 cells promoting IgG1 and IgE responses).
CD8+ T cell activation

The interaction of CD8+ T cells with MHC class I expressing cells is much less restricted than the interaction of CD4+ T cells with MHC class II molecules on professional APCs. Neglecting the two signal model of T cell activation, almost all peripheral cells could - theoretically - activate CD8+ T cells. Indeed, this has been documented in some experimental systems using e.g. fibroblasts to activate CD8+ T cells (Kündig et al., 1995) or tumour cell models where expression of costimulatory molecules on the tumour cells only enhanced immunogenicity but were not strictly required for the induction of CD8+ T cells (Chen et al., 1994). In line with this, it has been demonstrated that CTL responses in mice lacking IL-2 (Kündig et al., 1993b) or CD28 (Shahinian et al., 1993) were almost normal. On the other hand, non-lymphoid cells were shown to be unable to prime CD8+ T cells unless costimulatory signals such as IL-2, IL-4 or B7 were provided (Chen et al., 1992; Fearon et al., 1990; Golumbek et al., 1991; Harding and Allison, 1993; Ramarathinam et al., 1994; Townsend and Allison, 1993). As recently suggested by Kündig et al. for the induction of anti-viral CD8+ T cell responses in CD28-deficient mice (Kündig et al., 1996b), the strength and duration of signal 1 might be crucial in determining whether or not signal 2 is required for CD8+ T cell activation. An adequately longlived signal 1 seemed to be provided after infection with a replicating virus whereas administration of the relevant MHC class I binding peptide could not induce priming of CD8+ T cells unless continuously provided for a long time period.

However, the fact that the organized microenvironment of secondary lymphoid organs plays a crucial role in the successful activation of CD8+ T cells as illustrated by Karrer et al. (Karrer et al., 1997) should not be neglected. The importance of localized antigen presentation in secondary lymphoid organs has also been strengthened by Kündig et al. (Kündig et al., 1995) demonstrating that costimulation-deficient fibroblasts transfected with an immunogenic protein of viral origin could induce strong CD8+ T cell responses only if the fibroblasts were allowed to migrate into secondary lymphoid organs. Not only the organized structure of lymphoid organs plays a crucial role for the induction of T cells with non-professional APCs - the migration pattern of naive T cells may be equally important. Naive T cells, as opposed to activated T cells, reach the lymphoid organs via high endothelial venules. They do not migrate through tissue and therefore usually do not encounter antigens outside lymphoid organs (Jalkanen et al., 1986).
B 4. Lymphocyte effector functions

CD4+ T cell effector functions

Th cell functions are diverse and can be divided into two major categories: cognate Th cell-B cell interactions required for B cell activation and B cell isotype switch and T helper cell functions mostly mediated by the secretion of soluble mediators which may be directly involved in the control of infections by various pathogens and which may be important for DTH reactions, for the activation of macrophages, cytotoxic T lymphocytes and NK cells. More recently - as mentioned in the previous chapter - these different Th cell functions have been attributed to discrete Th cell subpopulations (Th1/Th2) with differing cytokine secretion patterns. Th cell functions can experimentally be assessed by in vitro proliferative responses to recall antigens and by the determination of concomitant cytokine release or more indirectly by the analysis of isotype-switched specific antibody responses.

Cytolytic CD4+ T cells can be generated by a number of protocols and MHC class II-restricted cytotoxic activity has been measured in vitro (Ju et al., 1988; Ozaki et al., 1987; Tite and Janeway, 1984). But it is still a matter of debate whether or not CD4+ T cells mediate - in analogy to CD8+ T cells - lytic effector functions in vivo or whether they exert an immunoregulatory function. CD4+ T cell-mediated cytotoxicity has been shown to be mediated by fas-fasL interactions (Ju et al., 1994; Stalder et al., 1994) between the Th cell effector (fasL positive) and the target cell (fas positive). Cytolytic CD4+ T cells have been demonstrated after influenza virus, measles virus and under certain circumstances after LCMV infections in vivo (Carreno et al., 1992; Eljaafari et al., 1992; Freer and Senesi, 1993; Jacobson et al., 1988; Maimone et al., 1986; van Binnendijk et al., 1989; van Binnendijk et al., 1992). (Muller et al., 1992). However, it remains unclear whether this in vitro measured lytic effector function of CD4+ T cells is representative for anti-viral immunological effector functions in vivo. Direct cytotoxic effector functions of in vivo relevance are clearly dependent on CD8+ T cell effectors as will be discussed in the next chapter.

Biologically more relevant assessments of CD4+ T cell effector functions are performed in vivo: Th cells play a central role in the activation and isotype-switch of antigen-specific B cells (Lanzavecchia, 1985; Singer and Hodes, 1983). After a viral infection, B cells recognize native viral antigens and are processing them similarly to macrophages and DC. Cognate interactions between B cells presenting the adequate Th cell epitope on MHC class II molecules and epitope-specific Th cells (the Th cell being previously activated by DC or macrophages - or maybe even by the B cell itself) results finally in the activation of the B cell: their proliferation, their differentiation into Ig-secreting plasma cells and isotype-switch of the anti-viral
antibody. Molecular interactions which are required to drive this activation process involve i) crosslinking of the surface Ig-receptors on the B cell (Parker, 1993), ii) MHC/peptide-TCR engagement (cognate T-B interaction), iii) CD40-CD40L interaction (Foy et al., 1993; Noelle et al., 1992)(as demonstrated for viral antigens in chapter 3 of the results section) and iv) the secretion of cytokines by the Th cell. This cognate interaction between B cells and Th cells does not predominantly occur in B cell follicles nor in germinal centers but rather in the outer periarteriolar sheath (PALS), i.e. in the outer T region (Van den Eertwegh et al., 1993). The CD40L molecule expressed on activated Th cells is indispensable for the generation of germinal centers (Foy et al., 1994).

An important exception to the mechanism of B cell activation described before are the T-independent B cell antigens (TI antigens) which are usually highly repetitive organized structures or alternatively polyclonal B cell activators (Bachmann et al., 1995; MacLennan and Gray, 1986). Whatever the nature of the B cell antigen, CD4+ T cells are mandatory in helping B cells to switch from IgM to other isotypes and to establish B cell memory (Burns et al., 1975). Many viral infections induce virus-specific (binding or neutralizing) antibodies of the IgG2a isotype which correlates well with the finding that viruses mostly induce Th1-like Th cell responses.

Th cells also have been shown to mediate anti-viral effector functions not only via the induction of protective antibody responses but also by the release of anti-virally active cytokines. Both IFNγ and TNFα have been attributed antiviral effector functions in various virus infection (Binder and Kündig, 1991; Glasgow, 1971; Hennet et al., 1992; Klavinsksis et al., 1989; Kündig et al., 1992; Leist et al., 1989a; Morris et al., 1982; Ramshaw et al., 1987; Wheelock and Toy, 1973; Wille et al., 1989). One key role of IFNγ is the prevention or reduction of host cell infection. The crucial involvement of IFNγ in protective immunity has been shown for vaccinia or listeria infections (Huang et al., 1993). Furthermore, Th-dependent activation of macrophages by IFNγ directly or by IL-2 indirectly may contribute to immune protection against vaccinia infection. IFNγ also increases MHC class I and II expression on APCs thereby influencing antigen presentation (Collins et al., 1984).

The degree by which anti-virally effective cytokines contribute to protective immune responses differs from one virus to the other, vaccinia virus infection being one example where anti-virally effective cytokines play a central role (Ramshaw et al., 1992).

More generally, the contributions of different effector cell types, of antibodies and soluble mediators in eliciting a protective immune response after a viral infection have to be investigated for each virus separately; the cytopathogenicity of a virus may correlate inversely with the strict requirement of CD8+ T cell effectors and directly with the requirement of an efficient humoral immune response. As shown for the
infection with non-cytopathic lymphocytic choriomeningitis virus (LCMV), perforin-mediated lysis of infected cells in vivo is of key importance in the successful clearance of the virus (Kägi et al., 1994a). On the other hand, neutralizing antibody responses are indispensable for protection after infection with the cytopathic vesicular stomatitis virus (VSV) (Dietzschold et al., 1987; Gobet et al., 1988; Lefrançois, 1984). As mentioned before, vaccinia virus infection is successfully controlled by anti-virally effective cytokines released by virus-specific T cells (Binder and Kündig, 1991). Other viral infections are not so exclusively controlled by a particular effector mechanism but rather by the interplay of CD8⁺, CD4⁺ T cell effector functions, antibody responses and soluble mediators (e.g. influenza and measles infections). The nature of the virus (cytopathic versus non-cytopathic) and the capacity of a virus to induce a certain effector function might be of importance in discriminating the effector cell type involved in protective immunity. For example LCMV elicits a very efficient CD8⁺ T cell response which is reflected by an extremely high precursor CTL frequency (1:3) 8 days after infection (Assmann-Wische et al., 1986). LCMV-specific Th cells are also induced but apparently much less pronounced as compared to the CD8⁺ T cell effectors. Challenge infection of an LCMV immune mouse with a recombinant vaccinia virus expressing a LCMV-derived protein is thus readily controlled by the CD8⁺ memory T cells (Binder and Kündig, 1991). If the initial induction of LCMV-specific CD8⁺ T cells hampered (e.g. in CD8-depleted mice), CD4⁺ T cells are dominantly induced and can now mediate protection against the challenge infection with the recombinant vaccinia virus. Chapter 4 of the results section investigates the role of various effector functions in the successful clearance of viral infections.

Whether or not and to what degree T help is required for the induction of anti-viral CD8⁺ effector cells is dependent on the virus. While T help is not necessary for the induction of anti-viral CD8⁺ effector T cells after some viral infections (e.g. LCMV) (Ahmed et al., 1988), CTL responses against vaccinia virus are nevertheless reduced in the absence of T help and CTL responses against VSV or influenza virus seem to depend even more on T help (Reiss and Burakoff, 1981). Thus, if CD8⁺ T cells are efficiently induced in high numbers they apparently can produce sufficient interleukins by themselves for induction, proliferation and effector cell functions.
CD8+ T cell effector functions

Cell contact-dependent cytolysis of target cells is the main effector function of CD8+ T cells and plays a key role in defence against intracellular pathogens such as viruses (Kägi et al., 1994a; Zinkernagel, 1993). On the other hand, cytotoxicity may cause pathology in rejection of foreign organ transplants, autoimmune diseases and immunopathology. As virus-specific cytotoxic T cells are induced upon infection with different viruses, it has been assumed that CD8+ effector T cells prevent virus proliferation by lysing infected cells. Theoretically, lysis of an infected cell by cytotoxic T cells could occur in the eclipse phase before any infectious virus has been produced and therefore could inhibit virus replication completely. Alternatively, cytotoxic T cells may reduce the production of infectious virus from an infected cell by lysing at a certain timepoint after assembly and release has already started. If recognition by cytotoxic T cells and lysis is relatively slow compared to viral cytopathic effects, then cytotoxic activity is effective only against non-cytopathic viruses, and the primary role of cytotoxic T cells is to eliminate and control otherwise persistently infected cells from the organism. Thus, the relative kinetics of cytotoxic T cell activity in vivo and the cytopathic or non-cytopathic character of a virus determines whether or not cytotoxic T cell activity is mandatory in virus control and protection.

Of key importance is the perforin-mediated target cell lysis mechanism where target cells are efficiently lysed by the release of preformed perforin molecules stored in granular structures of the CTL upon cognate interaction with its target cell. The released perforin molecules integrate and polymerize in the target cell membrane thus generating pores which interfere with the electrolyte balance of the cells with its surrounding and which allow the entry of granzymes into the target cell inducing apoptosis (Henkart, 1985; Kägi et al., 1996).

A more recently described cytolysis mechanism upon cognate cell interaction is dependent on the fas-fasL interaction where apoptosis is induced in the fas+ target cell upon interaction with a fasL expressing effector cell (Kägi et al., 1994b; Rouvier et al., 1993; Suda et al., 1993). Depending on the nature of the target cell type, this second mechanism might also contribute to lysis of virus-infected target cells but more importantly it has been suggested that fas-fasL interaction induced apoptosis might be crucial in the downregulation of T cell responses after the effector cell phase and in T cell homeostasis (Crispe, 1994; Lynch et al., 1995; Nagata and Golstein, 1995).

The prime importance of perforin-mediated cytolysis after LCMV infection in vivo has been clearly demonstrated using perforin-deficient mice (Kägi et al., 1994a). Especially important after infection with non-cytopathic viruses, CD8+ effector T
cell-mediated destruction of the virus-producing cell is the most efficient mechanism of clearing the virus from the mandatory host cell. This direct killing mechanism might not play such a dominant role in the control of cytopathic viruses which destroy the host cell and thus may be more efficiently controlled by anti-viral antibody responses.

B 5. Immunological memory

Immunological memory is responsible for the fact that secondary immune responses to T-dependent antigens are more effectively and more rapidly induced than primary immune responses. Immunological memory is established for both T and B cells (Gray and Sprent, 1990; Kündig et al., 1996a; Mackay, 1992; Sprent, 1994; Zinkernagel et al., 1996b) and usually long lasting as experienced by life-long immunity to childhood diseases and successfully performed vaccination campaigns. This mostly reflects longevity of B cell memory because protection against (re)infection is efficiently mediated by antibodies (Mims, 1987). The nature of protective T cell memory is still a matter of debate and dependent on the experimental read-out systems.

In many experimental situations, memory responses are at least partly a reflection of an increased precursor cell frequency. After primary exposure to an antigen, lymphocyte activation leads to a rapid expansion of antigen-specific cells and finally to the generation of effector cells. Most of these effector cells represent dead-end stages of lymphocyte development and they usually die within a few days due to lack of stimulation possibly because the antigen has been eliminated. However, not all activated lymphocytes will eventually be eliminated but some specific cells will remain in the antigen-experienced individual at an elevated precursor cell frequency. These cells differ in addition from naive cells by their altered expression of certain surface markers (CD44, L-selectin, CD45RO/RA isoforms), by different recirculation patterns and different life spans (Sprent, 1993; Tough and Sprent, 1994). For the establishment and the maintenance of both T cell and B cell memory, two different mechanisms have been suggested to be relevant: i) persistence of antigen due to a continuous infection, reexposure to the same or cross-reactive infectious agents, reexposure to antigen preserved in depots over long periods of time would all lead to continuous restimulation of antigen-specific lymphocytes and therefore would allow a
persistent low-level immune response or ii) the existence of a qualitatively special 'true' memory T or B cell which is independent of persisting antigen.

**T cell memory**

The cellular definition of a memory T cell has proven to be difficult since no special histological site has been described where memory T cells would be generated and since no general memory marker is known yet (as e.g. the expression of isotype-switched surface Ig in B cells) although a low expression of CD45RA and L-selectin as well as a high expression of CD44 have been attributed to a memory T cell status. But these surface markers are also expressed by activated T cells or effector T cells and thus serve only to distinguish all these developmental cell stages from naive T cells. On the other hand, it is also difficult to define a timepoint after antigen exposure where only memory cells would be present and all activated or effector T cells would have disappeared - especially if the antigen is still present in low amounts able to continuously restimulate T cells and thus a memory T cell may not be different from an activated T cell. In addition to the problem of phenotypically tracing memory T cells, the experimental read-out systems assessing memory T cell functions differ by their biological demands: T cell memory assessment by the usually applied *in vitro* tests might yield conflicting results as compared to assessment of protective T cell memory *in vivo*. Whereas *in vitro* assessment of memory T cells mostly reflects precursor T cell frequencies, *in vivo* protective capacity of memory T cells is biologically more relevant and demands most probably both an elevated T cell precursor frequency and an activated status of at least a fraction of these cells. In vitro measurable precursor T cell frequencies remain stable independently of the presence or absence of antigen and these elevated frequencies were shown to control virus replication in the spleen during a secondary infection (Doherty et al., 1994; Lau et al., 1994; Müllbacher, 1994). In contrast, T cell mediated protection against reinfection of peripheral solid organs was shown to be dependent on an activated status of the memory T cells and on antigen persistence (Kündig et al., 1996). The activation status of memory T cells seems to be particularly important for efficient protection of peripheral infections.

These findings suggest that no 'qualitatively' special memory T cell exists and that elevated precursor T cell frequencies together with constantly reactivated T cells are the consequence of clonal expansion and/or constant restimulation by persisting antigen.

The mechanisms by which the antigen might persist after infection are probably very different for the antigen-pool able to restimulate CD4+ T cells or CD8+ T cells. For
CD4\(^+\) T cells there is good evidence that antigen can be trapped in the form of immune complexes on follicular dendritic cells (FDCs) in germinal centers for months or perhaps years (Bachmann et al., 1996; Mandel et al., 1980; Tew and Mandel, 1979). These complexes have been suggested to be continuously processed by B cells which present then the relevant epitopes to CD4\(^+\) T cells (Gray et al., 1991; Kosco-Vilbois et al., 1993). In the case of CD8\(^+\) T cells there is no evidence that FCD-trapped antigen can be processed in order to be finally presented on MHC class I molecules. A more attractive view is that viral clearance after the primary immune response might not be absolute and that small numbers of cells might stay infected and thus continuously restimulate CD8\(^+\) T cells. These continuously activated CD8\(^+\) T cells have a critical role of constantly immuno-surveilling these foci of infection and of keeping them as small and limited in number as possible (Zinkernagel et al., 1996a).

Although even trace amounts of residual antigen might be capable of providing low-level stimulation of memory T cells, it has been also suggested that restimulation occurs via contact with cross-reactive environmental antigens (Beverley, 1990; Nahill and Welsh, 1993).

**B cell memory**

B cell responses can be divided into two phases, in an early phase where most antigen-specific B cells are not yet located in germinal centers but rather in B cell foci mostly as plasma cells (Jacob et al., 1991). In a later phase the foci disappear and the B cells are found in germinal centers. As opposed to plasma cell foci, germinal center B cells contain many hypermutations in their variable region genes and are thought to be progenitors of memory B cells (Jacob et al., 1991; McHeyzer-Williams et al., 1993). It is not yet clear whether B cells in foci and germinal center B cells derive from two different precursor B cell populations or not. Nevertheless, memory B cells are generated within the structures of the germinal center. Concerning the phenotype of memory B cells, they have been described as isotype-switched, long-lived, non-dividing, Th-independent cells circulating throughout the body not requiring interactions with persisting antigen for their survival (Bachmann et al., 1994c; Gray and Sprent, 1990; Schittek and Rajewsky, 1990; Vieira and Rajewsky, 1990).

Besides the presence of isotype-switched memory B cells, B cell memory is also characterized by elevated levels of specific antibodies. These memory antibody titers present at the time of secondary exposure to a certain pathogen are the most powerful strategy to cope with secondary infections since they may neutralize a given pathogen even before it infects a host cell (Mims, 1987; Steinhoff et al., 1995). The presence of
specific antigen which persists on follicular dendritic cells (FDCs) and is thus readily accessible for B cells seems to play a crucial role for the maintenance of such memory Ig titers.

However, it remains controversial whether or not the presence of persisting antigen is required for the survival of memory B cells. While some studies found memory B cells to be short-lived in the absence of persisting antigen (Celada, 1971; Gray and Skarvall, 1988) other investigations suggested an antigen-independent type of memory B cell (Schittek and Rajewsky, 1990) which is not - or only minimally - proliferating. In this line, no accumulation of specific memory B cells at the sites of antigen-persistence have been shown after local viral infections. In addition, after acute infections in the absence of adjuvants or preexisting IgG antibodies memory B cells were shown to freely recirculate throughout the lymphatic system independently of the localization of persisting antigen. This excluded a strict association between memory B cells and persisting antigen but showed nevertheless a strict colocalization of antigen and B cells undergoing full maturation to plasma cells (Bachmann et al., 1994c).

One reason that might account for some of these discrepancies may be the fact that not always a clear distinction between memory B cells and antibody forming cells (AFCs) was made. The former recirculate freely throughout the lymphatic tissue and the latter are restricted to sites of antigen persistence and the bone marrow and are responsible for the maintenance of the memory antibody titers (Bachmann et al., 1994c; MacLennan, 1994; Tsiagbe et al., 1992). Since most of these AFCs have a rather short life-span and thus have to be generated constantly from the pool of memory B cells, 2 distinct sets of memory B cells have been proposed: one population of recirculating B cells that does not proliferate and is detected in classical adoptive transfer experiments and a second population of memory B cells that remains associated with persisting antigen in germinal centers, proliferates and differentiates into AFCs (Bachmann et al., 1996). These AFCs then either migrate to the red pulp/marginal zone or eventually to the bone marrow.

This suggested existence of two distinct B cell populations efficiently contributing to B cell memory parallels the two distinct T cell populations efficiently contributing to T cell memory (as discussed in the last chapter): The presence of freely recirculating memory T cells and of freely recirculating memory B cells seems to be independent of persisting antigen (Bachmann et al., 1996; Hou et al., 1994; Lau et al., 1994) whereas activated memory T cells capable of recirculating through tissue and mediating protective immunity in the periphery seem to be dependent on restimulation by persisting antigen (Kündig et al., 1996) and, on the other hand, persisting antigen seems to be required for the continuous differentiation of memory B cells into AFCs thus maintaining protective antibody levels.
2. RESULTS

Chapter 1:

Presentation of endogenous viral proteins in association with MHC class II: on the role of intracellular compartmentalization, invariant chain and TAP transporter system

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Abbreviations used in this paper: APC, antigen presenting cell; B-GP, LCMV-GP expressed by recombinant baculovirus; B-NP, LCMV-NP expressed by recombinant baculovirus; P13, peptide 13; P61, peptide 61; ER, endoplasmic reticulum; GP glycoprotein; HEL, hen egg lysozyme; IL-2, interleukin 2; LCMV, lymphocytic choriomeningitis virus; Ii, invariant chain; MOI, multiplicity of infection; NP, nucleoprotein; pfu, plaque forming units
Abstract. MHC class II-associated antigen presentation is mainly linked to processing of exogenous antigens upon cellular uptake by endocytosis but has also been observed for endogenously expressed antigens. We have studied the MHC class II-associated presentation of the endogenously synthesized membrane associated glycoprotein (GP) and the cytosolic nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) in professional antigen presenting cells of mice. Since LCMV is a noncytopathic virus and minimally affects cellular protein synthesis, it is a convenient virus to study antigen presentation. In contrast, most other studies assessing class II-associated presentation of endogenously synthesized viral antigens used cytolytic viruses such as vaccinia, measles and influenza virus, which drastically interfere with host cell functions. In addition, most studies were performed using non-professional antigen presenting cells. We found that class II-associated presentation of endogenously synthesized membrane associated LCMV-GP was efficient and could not be inhibited by chloroquine or leupeptin and both the TAP transporter system and the invariant chain were not significantly involved in this process. In contrast, MHC class II-associated presentation of endogenously synthesized cytosolic LCMV-NP was not observed even in Ii-deficient antigen presenting cells. Thus, MHC class II loading of endogenously synthesized LCMV-GP apparently does not require processing in acidic endosomal compartments as defined by chloroquine and leupeptin insensitivity. Furthermore although the TAP molecules transport peptides of up to 15 amino acids length which potentially could bind to MHC class II molecules in the ER, such a process apparently does not occur for both the glyco- and nucleoprotein. Therefore, the subcellular localization of an endogenously synthesized protein influences crucially whether or not MHC class II loading can occur independently of the acidic compartments usually involved in MHC class II loading.
Introduction

The T-cell receptor of CD8+ and CD4+ T-cells recognizes short peptides derived from nominal antigens in association with MHC class I or II molecules, respectively (Babbitt et al., 1985; Braciale, 1992; Davis and Bjorkman, 1988; Unanue, 1992). Despite the structural and functional similarity between the MHC class I and class II molecules (Bjorkman et al., 1987a; Brown et al., 1993) they exhibit a different cellular distribution and follow different intracellular trafficking to the cell surface. Classically MHC class I molecules present endogenous antigens (i.e. endogenously synthesized proteins) whereas MHC class II molecules present antigen-fragments generated after uptake of exogenous antigens by endocytosis (Bevan, 1987; Germain, 1986; Moore et al., 1988; Morrison et al., 1986; Sweetser et al., 1989).

MHC class I molecules consist of a heavy chain that associates with β2 microglobulin in the endoplasmic reticulum (ER) where this heterodimer is loaded with peptides, mainly originating from endogenously synthesized proteins (Nuchtern et al., 1989; Townsend et al., 1989; Yewdell and Bennink, 1989). These bound peptides are generated in the cytoplasm most probably by the proteolytic action of the proteasome complex (Ciechanover, 1994). They are transported into the ER via the TAP transporter molecule (Braciale, 1992). Whether, and to what extent, the peptides are additionally trimmed within the ER in order to fit into the peptide binding groove of MHC class I is being debated (Bacik et al., 1994; Link Snyder et al., 1994; Roelse et al., 1994).

The heterodimeric MHC class II molecules consist of an α- and β-chain which associate in the ER with the invariant chain (li) (Cresswell, 1985; Cresswell, 1992; Guagliardi et al., 1990; Harding et al., 1989; Lamb et al., 1991; Neefjes et al., 1990; Peters et al., 1991). The invariant chain is thought i) to prevent the loading of newly synthesized class II molecules with peptides in the ER to some extent and ii) to contain an endosomal targeting signal and therefore to be important in directing MHC class II intracellular trafficking pathways distinctly from class I pathways (Bakke and Dobberstein, 1990; Germain, 1994; Sant and Miller, 1994; Stockinger et al., 1989). MHC class II molecules are loaded primarily but not exclusively (Castellino and Germain, 1995) with peptides within the recently described endosome-related acidic MIIC compartment which was characterized in B-cells and in MHC class II positive melanoma cells (Amigorena et al., 1994; Tulp et al., 1994). These peptides are generated by acidic proteolysis of endocytosed proteins in endosomal compartments. Although this MHC class discrimination by the immune system has been found to be very efficient, it has proven less stringent than initially assumed. There have been many reports describing MHC class I loading by exogenous antigens in vivo such as for ovalbumin, hepatitis B surface antigen, HIV-1 envelope protein, SV40 large T antigen, VSV nucleoprotein and LCMV glyco- and nucleoprotein (Bachmann et al.,
On the other hand, many endogenously synthesized proteins such as immunoglobulin light chains, measles virus matrix protein, influenza A matrix protein, vaccinia recombinant proteins, hen egg lysozyme, herpes surface antigen and myelin basic protein were found to be presented by MHC class II molecules (Brooks et al., 1991; Dodi et al., 1994; Jacobson et al., 1989; Jaraquemada et al., 1990; Jin et al., 1988; Malnati et al., 1992; Nuchtern et al., 1990; Sekaly et al., 1988; Weiss and Bogen, 1989; Weiss and Bogen, 1991). While some of these endogenous antigens were shown to be presented by an endosomal compartment-associated pathway defined by chloroquine-sensitivity (Jaraquemada et al., 1990; Jin et al., 1988; Malnati et al., 1992), some other endogenous antigens seemed to use other or additional pathways for MHC class II loading (Nuchtern et al., 1990; Sekaly et al., 1988).

We investigated MHC class II-associated presentation of two endogenously synthesized viral antigens with different subcellular localization in professional antigen presenting cells. These cells are suitable to study class II-associated antigen presentation, since they possess the recently described MIIC compartment which is involved in MHC class II loading (Amigorena et al., 1994; Tulp et al., 1994). LCMV is neither cytolytic nor inhibits host cell protein synthesis significantly and LCMV replication only minimally affects the host cell (Buchmeier et al., 1980; Lehmann, 1971). Therefore LCMV-infected professional APCs represent an ideal experimental system to analyse class II-associated presentation of endogenously synthesized viral antigens since subcellular compartments remain fully intact during infection. This confers an advantage of the LCMV model infection as compared to the cytopathic influenza, vaccinia and measles viruses used so far to study class II loading pathways of endogenously synthesized antigens.

I-A\(^b\)-associated presentation of LCMV-GP and NP-derived peptides was assessed by measuring induced IL-2 secretion of a GP- and a NP-specific CD4\(^+\) T-cell hybridoma, respectively. These peptides are involved in a primary anti-LCMV immune response as shown by both induction of peptide specific CD4\(^+\) T cells in vivo upon LCMV infection and for activation of the T cell hybridomas by in vivo infected APCs. We found that only endogenously synthesized LCMV-GP could load MHC class II molecules independently of proteolysis in acidic compartments as defined by chloroquine- and leupeptin insensitivity, whereas endogenously synthesized LCMV-NP failed to be presented independently of proteolysis in endocytic compartments even in the absence of Ii. These data indicate that processing and MHC class II loading of the endogenously synthesized membrane associated glycoprotein is likely to take place in the ER or pre-Golgi compartment. They suggest further that Ii may not compete with peptide binding to newly synthesized class II molecules in the ER but possibly interfere with binding of whole proteins.
Materials and Methods

Mice.
C57BL/6 mice were obtained from the breeding colony of the Institut für Zuchthygiene, Zürich. The generation of the mice deficient for the invariant chain and deficient for the TAP transporter system, respectively, has been described previously (Van Kaer et al., 1992; Viville et al., 1993). Mice were bred in a conventional mouse facility.

Viruses
The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube, Hamburg, Germany and grown on L929 cells with a low multiplicity of infection. The recombinant baculoviruses expressing the glycoprotein or the nucleoprotein of the LCM-virus have been described (Matsuura et al., 1987). All recombinant baculoviruses were derived from nuclear polyhedrosis virus and were grown at 28°C in Spodoptera frugiperda cells in TC-100 medium (Gibco Basel, Switzerland). Proteins were produced as described in (Bachmann et al., 1994a).
Recombinant vaccinia viruses expressing LCMV-GP, LCMV-NP and carboxyterminally truncated GP- and NP-proteins, respectively, have been described (Schulz et al., 1989; Whitton et al., 1988).

Generation of LCMV-specific Th-cell hybridomas
T-cell hybridoma VE8: CD8-deficient mice (Fung-Leung et al., 1991b) were immunized with 20 μg of purified, UV-inactivated LCMV-WE in incomplete Freunds adjuvants subcutaneously. Fourteen days later, lymph node cells were restimulated in vitro with 5μg/ml of purified, UV-inactivated LCMV-WE and irradiated C57BL/6 spleen cells. After 4 days activated T-cells were fused with the BW α-β- cell line (White et al., 1989). The specificity of hybridoma VE8 was characterized as follows: IL-2 secretion by the hybridoma was measured upon stimulation with irradiated spleen cells and different exogenous antigens such as UV-inactivated LCMV-WE, recombinant baculovirus-derived LCMV-GP or -NP. IL-2 in the supernatant was determined after 24 hours by the IL-2-sensitive cell line CTLL-2 followed by AlamarBlue™ (Biosource, International) colour reaction. VE8 is LCMV-NP-specific and I-A\(^b\) restricted. Peptide mapping of the VE8 hybridoma was performed in two steps. In a first step the protein region containing the VE8 epitope was defined using 5 different carboxyterminally truncated LCMV-NPs of different length as exogenously supplied antigens expressed by recombinant vaccinia viruses (Schulz et al., 1989). This experiment allowed to narrow down the region containing the epitope.
to about 100 amino acids of length. Finally the peptide mapping was performed with a series of overlapping 20-mer peptides. The peptide P61 recognized by VE8 consists of amino acids 309-328 of the nucleoprotein of LCMV-WE.

T-cell hybridoma 5A1: C57BL/6 mice were infected intravenously with 5x10^6 pfu of a vaccinia LCMV-GP recombinant virus. Twelve days later spleen cells were restimulated in vitro with 5μg/ml of UV-inactivated, purified LCMV-WE for 4 days followed by fusion with the BWα-β cell line (White et al., 1989). 5A1 specificity was determined in analogy to the VE8 hybridoma. 5A1 is I-A^b-restricted and specific for the LCMV glycoprotein. Initial epitope mapping was again performed using a series of carboxyterminally truncated vaccinia LCMV-GP recombinant viruses (Whitton et al., 1988) as exogenous antigens. The final peptide mapping with synthetic, overlapping peptides revealed the recognized peptide P13 consisting of amino acids 61-80 of the LCMV glycoprotein.

T-cell proliferation
C57BL/6 mice were immunized with 200 pfu of LCMV-WE intravenously. After 13 days CD4^+ T-cells were purified from a spleen cell suspension by MACS sorting. 1x10^5 CD4^+ T-cells were incubated in 96-wells with 3-fold serial dilutions of either purified, UV-inactivated LCMV (highest conc.:5 μg/ml), P13 or P61 (highest conc.: 5 μg/ml) or medium only in the presence of 7x10^5 irradiated (2000 cGy) C57BL/6 spleen cells for 3 days. Proliferation was assessed by incorporation of ^3H-thymidine (25 μCi/well).

Peptide presentation by in vivo infected APCs
C57BL/6 mice were immunized with 2x10^6 pfu LCMV and 6 days later spleen cells from infected, from naive and from LCMV-carrier mice were irradiated (2500 cGy) and incubated in 3-fold serial dilutions (highest conc.: 3x10^6 cells /well) together with 4x10^4 5A1 or VE8 T-cell hybridomas, respectively for 20 hrs. IL-2 concentration in the supernatant was determined as described above.

MHC class II presentation of viral antigens in the presence or absence of chloroquine or leupeptin
In the absence of drugs 2x10^5 lymphocytes from either C57BL/6, Ii-deficient or TAP-deficient mice were incubated with 3-fold serial dilutions of live LCMV (MOI=2 at the highest concentration), UV-inactivated LCMV (same amount as live LCMV), recombinant baculovirus-derived LCMV-GP or LCMV-NP (3μg/ml at the highest concentration), P13 (1μg/ml at the highest concentration) or P61 (1μg/ml at the highest concentration) in 96-wells in a total volume of 50 μl. After 4 hours 4x10^4 hybridoma cells per well were added in a volume of 150 μl for 13 hours. Then 50 μl
supernatant were transferred into a new 96-well and 1x10^4 CTLL cells were added per well for 15 hours. Quantification of viable CTLL cells was performed by AlamarBlue™ colour reaction (Biosource, International) and measured by fluorescence emission at 590 nm using the CytoFluor™ 2350 (Millipore) fluorimeter. If the antigen presentation assay was performed in the presence of chloroquine or leupeptin, the APCs were incubated with the same serial dilutions of antigens in the same volume as above but containing 150 μM of chloroquine or 0.5 mM leupeptin for 4 hours. The 4x10^4 hybridoma cells were added in a volume of 150 μl for 13 hours lowering the chloroquine concentration to 38 μM. In the leupeptin experiments 4x10^4 hybridoma cells were added in a volume of 150 μl containing 0.5 mM leupeptin. IL-2 concentration in the supernatant was determined as described above.

MHC class II presentation of regurgitated antigens
200 μl of the same supernatants tested for IL-2 content as described above or 5 μg/ml peptide were incubated for 3 hrs with 7x10^6 C57BL/6 spleen at 37°C. Subsequently the cells were washed three times and incubated over night together with 4x10^4 hybridoma cells. IL-2 concentration in the supernatant was determined as described above.
Results

Generation and characterization of two I-A\textsuperscript{b} restricted T-cell hybridomas
Immunization of naive mice with UV-inactivated, purified LCMV or recombinant vaccinia-virus expressing LCMV-GP and subsequent restimulation in vitro with purified, UV-inactivated LCMV resulted in two LCMC-specific T-cell hybridomas 5A1 and VE8, recognizing LCMV-GP and LCMV-NP, respectively (Fig.1). Epitope mapping revealed a GP-derived peptide P13 (amino acids 61-80 of LCMV-GP) and a NP-derived peptide P61 (amino acids 309-328 of LCMV-NP) (see materials and methods for details) (Fig.1A). The sensitivities for exogenously supplied LCMV-antigen of both hybridomas were in a comparable range shown by the equal IL-2 production upon stimulation with graded amounts of purified, UV-inactivated LCMV (Fig.1B).
Fig. 1 (A) Antigen specificity of T-cell hybridomas 5A1 and VE8. C57BL/6 spleen cells were pulsed with different exogenously supplied antigens (UV-inactivated purified LCMV, baculovirus recombinant LCMV glycoprotein or nucleoprotein and LCMV peptides P13 (GP) and P61 (NP)) and activation of the T-cell hybridomas 5A1 and VE8 was measured by secretion of IL-2 using the IL-2 sensitive cell line CTLL. AlamarBlue™ colour reaction was used for quantification of viable CTLL cells. Exact concentrations of the stimulating antigens are described in the Materials and Methods paragraph. (B) The sensitivities of both hybridomas for exogenously supplied LCMV-antigen are in a comparable range. The sensitivities for exogenous LCMV antigen were compared using the same 3-fold serial dilutions of UV-inactivated purified LCMV and C57BL/6 spleen cells as APCs.
Presentation of P13 and P61 in vivo

We analysed whether P13 or P61 represented epitopes recognized during a normal LCMV-specific immune response. T-cell proliferation of purified CD4+ Th-cells 13 days post infection of C57BL/6 mice with 200 pfu LCMV clearly showed that both P13- and P61 specific Th-cells are induced in vivo (Fig.2A) demonstrating that during the natural time course of a LCMV-infection both P13 and P61 are presented and recognized.

Additionally we measured peptide presentation in vivo using irradiated spleen cells from LCMV-infected C57BL/6 mice 6 days post infection as APCs. Activation of both hybridomas was observed using these in vivo generated APCs (Fig.2). Therefore both hybridomas 5A1 and VE8 recognize peptides that are naturally generated in vivo upon LCMV-infection.
Fig. 2 (A) T-cell proliferation ex vivo. Purified CD4+ T-cells from a LCMV-infected mouse proliferated in the presence of either purified, UV-inactivated LCMV or the peptides P13 or P61, respectively. (B) Presentation of P13 or P61 in vivo. Irradiated spleen cells from a LCMV-infected mouse were used as APCs to activate the T-cell hybridomas 5A1 or VE8.
Activation of the GP and NP-specific hybridomas using replicating versus inactivated LCMV.

We examined the capacity of either LCMV infected C57BL/6 spleen cells or spleen cells loaded with exogenously supplied UV-inactivated LCMV to activate the GP-specific hybridoma 5A1 or the NP-specific hybridoma VE8 (Fig.3). Whereas in the case of the NP-specific hybridoma VE8, live and UV-inactivated LCMV exhibited the same activation capacity, live virus showed an at least 10-fold enhanced presentation capacity as compared to UV-inactivated virus in the case of the GP-specific hybridoma 5A1 (Fig.3). Thus, LCMV-NP seemed to be only presented via the classical endocytosis pathway whereas LCMV-GP apparently was also presented by an additional class II presentation pathway. A possible explanation for the enhanced MHC class II loading by LCMV-GP derived from live virus as compared to UV-inactivated virus could be, that the membrane associated GP was shed and degraded outside the cell surface. To rule out this external class II loading by GP-fragments, fresh APCs were incubated with overnight culture supernatant from LCMV infected APCs, from APCs pulsed with UV-inactivated LCMV and from APCs pulsed with freshly supplied peptide. Thereafter the cells were washed extensively and used as APCs to activate the T-cell hybridomas (Fig.4). Only spleen cells either incubated with newly added appropriate peptide were able to activate the T-cell hybridomas. Neither culture supernatant from LCMV infected APCs nor supernatant from APCs pulsed with UV inactivated LCMV could provide sufficient amounts of protein fragments for class II loading as evaluated by specific IL-2 release by sensitive T-cell hybridomas. Therefore the enhanced class II presentation of LCMV-GP derived from live virus could not be explained by regurgitation of presentable GP-derived protein fragments by the APC.

The possibility that the enhanced presentation of LCMV-GP was due to progeny virus that entered the endocytosis pathway could be excluded because i) the same effect should have been observable in that case for LCMV-NP presentation and ii) virus titers were determined in the supernatant and within the APCs at the end of the assay and were found to be less than about 1/60th of the initial virus dose (data not shown).
Fig. 3 Hybridoma responsiveness to live versus UV-inactivated LCMV. In the presence of C57BL/6 APCs live versus UV-inactivated virus were compared in their activation capacity of the 5A1 or VE8 T-cell hybridomas. Live LCMV and UV-inactivated LCMV were used to activate the GP-specific hybridoma 5A1 (A) or NP-specific hybridoma VE8 (B).

Fig. 4 Regurgitation and class II presentation of viral antigens. The same supernatants used in Fig.3 for measurement of IL-2 content or newly supplied peptides were used to pulse fresh C57BL/6 spleen cells. After extensive washing these spleen cells were used as APCs to stimulate the T-cell hybridomas. IL-2 production was determined with the IL-2 sensitive cell line CTLL.
Chloroquine resistance of MHC class II presentation of endogenously synthesized LCMV-GP

There have been described both a chloroquine-insensitive pathway for class II presentation of endogenously synthesized antigens in the case of measles virus, influenza matrix and endogenous \( \lambda \)-chain presentation (Nuchtern et al., 1990; Sekaly et al., 1988; Weiss and Bogen, 1991) and a chloroquine-sensitive pathway in the case of vaccinia-HA recombinant viruses and vaccinia-Hepatitis B surface antigen recombinant virus (Dodi et al., 1994; Jaraquemada et al., 1990; Jin et al., 1988; Malnati et al., 1992). We therefore analysed chloroquine sensitivity of class II presentation of endogenously synthesized LCMV-GP (Fig.5). In LCMV infected C57BL/6 spleen cells endogenously synthesized LCMV-GP was efficiently presented in the presence of chloroquine whereas exogenously supplied antigens were not presented (as shown here for the recombinant LCMV-NP, GP and UV-inactivated LCMV). In contrast, LCMV-NP was not presented at all in the presence of chloroquine. This is consistent with the notion that the nucleoprotein is only presented via the classical endocytosis pathway. Presentation of exogenously added appropriate LCMV-GP and -NP derived soluble peptides was only marginally affected by the presence of chloroquine.

The possibility that presentation of endogenously synthesized LCMV-GP is due to a much higher intracellular GP concentration or a higher sensitivity of the GP-specific T-cell hybridoma could be ruled out for the following reasons: i) the same virally infected APCs were used for the presentation of endogenous LCMV-GP or NP, ii) LCMV-NP is the first synthesized viral protein after infection (Buchmeier et al., 1978), iii) LCMV-NP is present at higher intracellular concentrations (75% viral protein mass) compared to the GP (25% viral protein mass) (Vezza et al., 1977) and iv) both T-cell hybridomas exhibited a comparable sensitivity towards exogenously supplied LCMV antigen (Fig.1). These findings suggested that the subcellular compartmentalization seems to play a critical role for the presentation of endogenously synthesized antigens.
Fig. 5 Chloroquine sensitivity of antigen presentation. The T-cell hybridomas 5A1 and VE8 were stimulated in presence or absence (same data as in Fig.1) of chloroquine with various antigens (live LCMV, UV-inactivated LCMV, baculovirus recombinant LCMV-GP and-NP, P61 and P13) in the presence of C57BL/6 spleen cells as APCs. IL-2 production was determined using the IL-2 sensitive cell line CTLL.
Leupeptin resistance of MHC class II presentation of endogenously synthesized LCMV-GP

Leupeptin has been shown to inhibit intracellular degradation of polypeptides in acidic compartments and thereby to deplete - at least to some extent - the endocytic pathway of peptides available for class II binding (Hitzel et al., 1995). Additionally proteolytic removal of Ii is inhibited by leupeptin as well as surface deposition of newly synthesized class II molecules (Neeffes and Ploegh, 1992). In analogy to the chloroquine experiments, only endogenously synthesized LCMV-GP was able to load MHC class II molecules in the presence of leupeptin but neither exogenous LCMV-GP or -NP antigen nor endogenously synthesized NP were able to load class II molecules in the presence of leupeptin (Fig.6).

Fig. 6 Leupeptin sensitivity of antigen presentation. Using C57BL/6 spleen cells as APCs the T-cell hybridomas 5A1 and VE8 were stimulated in the presence or absence of leupeptin with the same antigens as described in Fig.5. IL-2 production was determined with the IL-2 sensitive cell line CTLL.
Invariant chain is neither needed nor inhibitory for presentation of endogenously synthesized LCMV-GP

The invariant chain has been shown to be important for presentation of exogenously supplied antigens and is thought to prevent MHC class II loading with peptides in the ER (Bodmer et al., 1994; Loss and Sant, 1993; Sant and Miller, 1994). Therefore, it was conceivable that LCMV nucleoprotein degraded in the cytosol (and transported into the ER e.g. by the TAP transporter) might be presented in the absence of Ii or that the LCMV membrane glycoprotein might be even better presented on MHC class II in the absence of Ii. Antigen presentation experiments were performed using spleen cells from invariant chain deficient mice as APCs (Fig.7A). In order to determine only class II presentation of endogenous proteins, the experiments were performed in the presence of leupeptin. MHC class II presentation of endogenously synthesized GP by C57BL/6 spleen cells or Ii-deficient spleen cells was comparable if not marginally enhanced in Ii-deficient APCs. Accordingly, peptide presentation on B6 APCs and on Ii-deficient APCs was comparable - if not enhanced on Ii deficient APCs - using non-saturating peptide concentrations (not shown).

In contrast, no MHC class II presentation of endogenously synthesized cytoplasmic nucleoprotein could be detected in Ii-deficient APCs in the presence of leupeptin (Fig.7A). As expected, no significant presentation of LCMV-NP was observed also in the absence of leupeptin (not shown). Therefore LCMV-NP protein fragments that might be present in the ER in C57BL/6 spleen cells were not able to bind to MHC class II also in the absence of competing Ii.

TAP transporter is not needed for presentation of endogenous LCMV glycoprotein

It has previously been suggested that MHC class II loading with peptides derived from endogenously synthesized proteins in the ER requires functional TAP transporters (Bijlmakers et al., 1994). Therefore, we analysed whether the absence of the TAP transporter influenced presentation of endogenous LCMV-GP or -NP by using spleen cells from TAP-deficient mice in the presence of leupeptin as APCs (Fig.7B). Presentation of endogenously synthesized LCMV-GP was not impaired in spleen cells from mice lacking the TAP transporter molecule. This result demonstrated that the import of GP (-fragments) presentable by MHC class II into the ER was not dependent on a functional TAP transporter but most probably occurred by cotranslational import into the ER. Presentation of endogenously synthesized NP was not affected by the absence of TAP transporter (Fig.7B), consistent with the finding that cytosolic LCMV-NP was not presented at all by a class II loading pathway independent of proteolysis in acidic compartments.
Fig. 7 Antigen presentation in Ii-deficient and TAP-deficient APCs. (A) Ii-deficient spleen cells as APCs: The same antigens as described in Fig.5 were used for stimulation of the T cell hybridomas 5A1 and VE8 using spleen cells from invariant chain deficient mice as APCs in the presence of leupeptin. (B) TAP deficient spleen cells as APCs: The same antigens as described in Fig.5 were used in the presence of leupeptin and of spleen cells form TAP-deficient mice as APCs in order to activate the T cell hybridomas 5A1 and VE8.
Discussion

This study describes TAP- and Ii-independent as well as chloroquine and leupeptine insensitive MHC class II presentation of endogenously synthesized membrane associated glycoprotein in LCMV-infected professional APCs. In contrast, endogenously synthesized LCMV-NP, a cytosolic protein, was not presented by this chloroquine and leupeptin insensitive class II loading pathway. Since the peptides studied are involved in a primary anti-LCMV immune response and since the noncytopathic LCMV does not interfere measurably with the cell physiology (Buchmeier et al., 1980; Fields, 1990; Lehmann, 1971), these results are biologically representative. They indicate that alternative albeit less efficient processing pathways exist whereby class II molecules may be loaded by endogenously synthesized proteins (Bijlmakers et al., 1994).

Many studies on MHC class II presentation of endogenously synthesized antigens have been performed earlier leading to apparently contradictory results concerning the mechanism(s) of intracellular MHC class II loading. Most of these results are summarized in Table 1 and are discussed in relation to our findings. In the upper section of Table 1 MHC class II presentation studies of endogenous self-antigens as well as of endogenous neo-self antigens are listed. MHC class II-associated presentation in professional APCs is almost exclusively observed for endogenously synthesized proteins either residing in or passing through the ER (Bikoff, 1992; Bodmer et al., 1994; Brooks et al., 1991; Chen et al., 1990; Moreno et al., 1991; Newcomb and Cresswell, 1993; Rudensky et al., 1991a; Rudensky et al., 1991b). In contrast, endogenously synthesized proteins which naturally do not have access to the ER are not found to be presented on MHC class II molecules. As an exception, endogenous measles virus-derived matrix and nucleocapsid proteins were found to be presented on MHC class II molecules (Jacobson et al., 1989). In some studies the presence of Ii proved to be inhibitory for MHC class II loading by endogenous antigens (Bodmer et al., 1994; Newcomb and Cresswell, 1993), whereas in other studies the presence of Ii seemed to be irrelevant for MHC class II loading by endogenously synthesized antigens (Bikoff, 1992; Bodmer et al., 1994; Jacobson et al., 1984). Therefore one could assume that certain antigens are not able to compete efficiently with Ii in the ER for binding to newly synthesized class II molecules or that certain endogenous antigens still are somehow able to load class II molecules via the classical endosomal class II loading pathway.
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<th>Reference</th>
<th>APC</th>
<th>TAPd</th>
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<th>H-2</th>
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TABLE 1. MHC class II-associated presentation of endogenously synthesized proteins related to their intracellular localization and to the nature of the antigen presenting cell.
Analysed epitope on depends on presentation II class on TAP of antigen.

Dependence on inhibition by BFA or Chloroquine of presentation only indicates when examined in the report.

\(...\)

Chloroquine sensitivity of class II presentation only indicates when examined in the report.

<table>
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<th>Reference</th>
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The notion that only endogenous proteins residing or passing through the ER are able to load class II molecules is corroborated by the findings of Weiss and Bogen (Weiss and Bogen, 1991), who targeted an endogenous λ-chain to different subcellular compartments. Only the transmembrane or ER-targeted proteins were presented on MHC class II whereas the cytosolic or nuclear proteins were not presented on class II. The same is true for differently targeted endogenous HEL antigens where only soluble, transmembrane or ER-targeted HEL was able to load MHC class II (Brooks et al., 1991). Additionally, it has also been demonstrated in one example (Chen et al., 1990) that MHC class II-associated presentation of the endogenous self antigen HLA-B/C is insensitive to treatment with chloroquine and therefore apparently does not require proteolysis in acidic compartments.

Class II presentation of infectious virus-derived endogenous antigens has been analyzed as listed in the lower section of Table 1; yet the data do not yield an easily generalisable picture. It is difficult to compare these results since firstly different and often non-professional APCs were used and secondly the different characteristics of the various viral systems do not permit valid comparisons. The importance of the nature of the APC has been revealed in two examples: 1. endogenous cytosolic V-CH3 was presented in fibroblasts but not in myeloma cells whereas a secreted V-CH3 protein was presented in both fibroblasts and myeloma cells (Bikoff, 1991; Bikoff, 1992) and 2. certain epitopes of transfected, membrane-associated HEL were presented in professional APCs but not in fibroblasts (Moreno et al., 1991).

MHC class II presentation of recombinant vaccinia virus derived endogenously synthesized proteins generally seems to be chloroquine sensitive irrespective of the subcellular localization of the proteins as shown for vaccinia-matrix (influenza) (Jaraquemada et al., 1990), for vaccinia-H3, vaccinia-cytoH3, vaccinia-miniH3 (Malnati et al., 1992) and vaccinia-hepatitisB surface antigen (Jin et al., 1988). This chloroquine-sensitive class II loading by endogenously synthesized membrane- as well as cytosolic proteins might be explained by the fact that vaccinia virus infection leads to a major shut-down in cellular protein synthesis resembling a treatment with Brefeldin A or cycloheximide (Fields, 1990). Therefore, membrane proteins such as influenza HA which are naturally transported into the ER might not be able to load MHC class II molecules outside of endosomal compartments in the presence of chloroquine because no newly synthesized αβ class II heterodimers are available. Thus, probably only recycled MHC class II molecules from the surface can be loaded with recycled HA from the membrane under these circumstances, a process which ought to be inhibitable by chloroquine. This recycling pathway is also used to explain the data by Polydefkis and colleagues. Using recombinant vaccinia viruses they found that only the endogenous membrane-associated form of HIV gp160 is presented on
class II, whereas the secreted or cytosolic forms of HIV gp160 were not presented (Polydefkis et al., 1990). Cytoplasmic proteins derived from vaccinia virus recombinants somehow seem to gain access to endosomal compartments possibly by degradation-promoting pathways (Ciechanover, 1994), by association with heat shock proteins (Schirmbeck and Reimann, 1994) or by the fact that vaccinia virus forms large intracellular aggregates that might fuse with endosomal compartments (Fields, 1990). Since vaccinia virus and similarly influenza virus are cytolytic, it is conceivable that vaccinia virus and influenza virus induce a breakdown of intracellular compartmentalization. This makes it very difficult, if not impossible, to compare processing of differently targeted recombinant proteins, since the proteins might finally be uniformly distributed intracellularly.

Our results, which resemble data obtained with transfected cell lines or data obtained by elution and analysis of natural self peptides bound to class II molecules (Bikoff, 1991; Bodmer et al., 1994; Brooks et al., 1991; Chen et al., 1990; Newcomb and Cresswell, 1993; Polydefkis et al., 1990; Rudensky et al., 1991a; Weiss and Bogen, 1991) therefore suggest that the contradictory results obtained with different viral systems do not reflect particular processing properties of professional APCs but rather reflect different effects of the viruses on host cell membrane compartments, protein synthesis and processing.

Upon LCMV infection of professional APCs, endogenously synthesized LCMV-GP was found to be loaded onto MHC class II molecules in a chloroquine- and leupeptin-insensitive manner. Additionally, class II presentation of endogenous LCMV-GP was equally observed in the absence if TAP and Ii - if not even enhanced in the absence of Ii. Since leupeptin and chloroquine may not abolish all lysosomal/endosomal proteolysis, our results do not formally prove that processing of endogenous GP and class II loading by endogenous GP occur outside endosomal compartments. Nevertheless, all together chloroquine-insensitivity, leupeptine-insensitivity and independence of a present or absent Ii favour the interpretation that endogenous LCMV-GP is processed and loaded onto class II molecules outside endosomal compartments - most probably within the ER. Upon cotranslational import of the LCMV-GP into the ER, the likely still linear polypeptide may directly associate with newly synthesized MHC class II αβ heterodimers. Within this trimolecular complex LCMV-GP has to efficiently compete with the binding of the invariant chain because peptide binding and Ii-binding have been shown to be mutually exclusive (Bijlmakers et al., 1994). The LCMV-GP in this trimolecular complex could be trimmed either within the ER or in a pre-Golgi compartment. It has in fact been shown that proteolytic activity may exist within the ER (Bacik et al., 1994; Link Snyder et al., 1994; Roelse et al., 1994). These MHC class II-peptide complexes would then be
transported directly to the cell surface by the secretory pathway (Bijlmakers et al., 1994).

The cytosolic LCMV-NP synthesized upon LCMV-infection, however, does not seem to be able to be loaded onto class II molecules in a comparable manner to LCMV-GP. Two different possible explanations may account for this finding: 1. It is conceivable that the protease machinery in the cytoplasm generates a different peptide repertoire than the proteases in the acidic endosomal compartments as shown by Bodmer et al. (Bodmer et al., 1994; Moreno et al., 1991). Accordingly, it may well be that the NP peptide recognized by the hybridoma VE8 is not generated by cytoplasmic proteases but is generated within the endosomal compartments. 2. NP-peptides are generated in the cytosol and transported into the ER via the TAP transporter system (it has been shown that TAP transporter molecules accept peptides of up to 15 amino acids length (Schumacher et al., 1994)), but that MHC class II molecules in the ER might not be able to bind these free peptides. This second possibility seems to be more likely and implies that Ii may not really prevent peptide binding to free MHC class II molecules in the ER but rather interferes with the binding of whole proteins.

The here described class II presentation pathway of endogenously synthesized LCMV-GP but not of LCMV-NP corroborates previous findings that at least some endogenously synthesized proteins that naturally have access to ER/pre-Golgi compartments are able to load MHC class II molecules most probably outside endosomal compartments. This pathway may actually be important for T-helper cell induction in vivo. Virus infected professional APCs may be able to initiate an antiviral immune response even before the infected cells are lysed - a pathway that may be particularly important for non-cytolytic viruses - and before viral antigens are liberated to enter the classical exogenous class II pathway in order to activate T-helper cells.
Chapter 2:

**Functional in vivo MHC class II loading by endogenously synthesized glycoprotein during viral infection¹**

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Abbreviations used in this paper: B-GP, LCMV-GP expressed by recombinant baculovirus; B-NP, LCMV-NP expressed by recombinant baculovirus; P13, peptide 13; P61, peptide 61; ER, endoplasmic reticulum; GP glycoprotein; LCMV, lymphocytic choriomeningitis virus; Ii, invariant chain; MOI, multiplicity of infection; NP, nucleoprotein; pfu, plaque forming units; VSV, vesicular stomatitis virus
Summary

MHC class II presentation of antigenic peptides derived from soluble proteins is usually preceded by antigenic uptake via (non-receptor-mediated) endocytosis by professional antigen presenting cells followed by processing in endosomal compartments. Although in vitro alternative pathways for MHC class II loading have been described for certain intracellularly synthesized proteins, the importance of these pathways has not been assessed in vivo. We have previously shown that endogenously produced membrane-associated glycoprotein of lymphocytic choriomeningitis virus (LCMV), a non-cytopathic virus, can be presented in vitro on MHC class II molecules in the absence of the invariant chain (Ii) whereas the cytosolic LCMV-nucleoprotein failed to be presented under the same conditions. Taking advantage of this system, we analyzed presentation of LCMV-glycoprotein and nucleoprotein in vivo in Ii-deficient mice and followed the induced T helper cell and B cell responses. At early timepoints after LCMV infection of Ii-deficient mice we found in vivo MHC class II loading exclusively by the endogenously synthesized LCMV-GP whereas no MHC class II loading by LCMV-NP could be detected. As a direct consequence, LCMV-specific Th cells exhibited initially only LCMV-GP specificity. In contrast, both LCMV-GP- and LCMV-NP-derived epitopes were presented in comparable amounts on APCs upon LCMV infection of normal mice and LCMV-GP as well as LCMV-NP-specific T helper cells were comparably induced in vivo.

Thus, alternative, cell internal MHC class II loading pathways are functional in vivo and may become dominant if the usual antigen presentation pathways are hampered.
Introduction
MHC class I and MHC class II loading have been shown to generally follow different processing pathways. MHC class I loading is mainly connected to proteasome-mediated cytosolic processing of endogenously synthesized proteins followed by peptide transport into the ER via the heterodimeric TAP transporter complex (Braciale, 1992; Ciechanover, 1994; Germain, 1986). Whether and to what extent these peptides are additionally trimmed within the ER is still a matter of debate (Bacik et al., 1994; Link Snyder et al., 1994; Roelse et al., 1994). In the ER peptides displaying appropriate lengths as well as appropriate anchor residues are bound to newly synthesized MHC class I molecules which are subsequently transported to the cell surface.

In contrast, newly synthesized MHC class II molecules associate in the ER with the invariant chain (Cresswell, 1985; Cresswell, 1992; Guagliardi et al., 1990; Harding et al., 1989; Lamb et al., 1991; Neefjes et al., 1990; Peters et al., 1991) or with other polypeptides (Busch et al., 1995) thereby forming a trimeric structure (Roche et al., 1991). The invariant chain has an important role in antigen presentation. It has been shown i) to inhibit peptide binding to MHC class II molecules in the ER, ii) to stabilize empty MHC class II molecules and iii) to direct transport of MHC class II molecules to acidic endosomal compartments (Bakke and Dobberstein, 1990; Germain, 1994; Sant and Miller, 1994; Stockinger et al., 1989). Peptide loading of MHC class II molecules occurs in these endosomal compartments after proteolysis of the invariant chain by a HLA-DM (or the murine equivalent H2-M)-catalyzed process exchanging the residual CLIP peptide with the antigenic peptides (Denzin and Cresswell, 1995; Roche, 1995; Sherman et al., 1995). The antigenic peptides are generated after endocytosis-mediated uptake of soluble proteins or protein complexes which are then processed in acidic endosomal compartments (Germain and Margulies, 1993; Harding et al., 1988; McCoy and Schwartz, 1988). Subsequently peptide-loaded MHC class II molecules are transported to the cell surface.

Several exceptions from this classical MHC class II loading pathway have been reported that demonstrated MHC class II loading by endogenously synthesized proteins (Aichinger and Lechler, 1995; Bodmer et al., 1994; Brooks et al., 1991; Chen et al., 1990; Dodi et al., 1994; Jacobson et al., 1989; Jaraquemada et al., 1990; Jin et al., 1988; Malnati et al., 1992; Moreno et al., 1991; Newcomb and Cresswell, 1993; Nuchtern et al., 1990; Oxenius et al., 1995; Pinet et al., 1994; Polydefkis et al., 1990; Rudensky et al., 1991a; Rudensky et al., 1991b; Sekaly et al., 1988; van Binnendijk et al., 1992; Weiss and Bogen, 1989; Weiss and Bogen, 1991). These studies may be divided into mainly three different groups of experimental systems: 1. Transfection experiments of APCs with genetically engineered recombinant proteins exhibiting different subcellular localizations revealed that some proteins having naturally access
to the ER are able to load MHC class II molecules independently of endosomal processing (Bikoff, 1992; Bodmer et al., 1994; Brooks et al., 1991; Jacobson et al., 1989; Moreno et al., 1991; Weiss and Bogen, 1991). 2. Elution of peptides naturally bound to MHC class II molecules on the surface of APCs demonstrated that these peptides were almost exclusively derived from either ER-resident, membrane or secreted proteins (Chen et al., 1990; Rudensky et al., 1991a; Rudensky et al., 1991b) and that some of these intracellularly synthesized proteins can load MHC class II molecules (Chen et al., 1990; Newcomb and Cresswell, 1993). A striking feature of these non-viral experimental systems is that the subcellular localization of proteins is crucially connected to the ability to load MHC class II molecules independently of endosomal processing. In a third group of experiments, MHC class II loading by endogenously synthesized proteins was analyzed upon viral infection of APCs (Dodi et al., 1994; Jaraquemada et al., 1990; Jin et al., 1988; Malnati et al., 1992; Nuchtern et al., 1990; Oxenius et al., 1995; Pinet et al., 1994; Polydefkis et al., 1990; Sekaly et al., 1988; van Binnendijk et al., 1992). In this last group of experiments, the intracellular localization requirements for an endogenously synthesized protein in order to load MHC class II molecules seemed to be much less defined. But a valid comparison to the first two experimental groups might be rendered difficult due to the differences each individual group of viruses exibits during infection of host cells.

We have previously shown in in vitro studies that upon infection of APCs with LCMV, endogenously synthesized membrane associated LCMV-GP is able to load MHC class II molecules independent of proteolysis in acidic compartments as defined by chloroquine- and leupeptin-insensitivity. In addition, the presence of the invariant chain was neither required nor inhibitory for efficient MHC class II loading by endogenously synthesized LCMV-GP (Oxenius et al., 1995). In contrast, cytosolic LCMV-NP was not able to load MHC class II molecules via an endosomal processing-independent pathway, thereby corroborating the findings in non-viral systems that the subcellular localization of a protein is of crucial importance for non-classical MHC class II loading.

The in vivo relevance of these various special pathways of antigen presentation has not been defined yet. Here we report that MHC class II loading by endogenously synthesized membrane associated LCMV-GP but not by cytosolic LCMV-NP is also observed in vivo after infection with LCMV. To differentiate between MHC class II loading by endogenously synthesized and exogenous antigens, Ii-deficient mice were used. In vivo infected APCs from Ii-deficient mice exclusively presented a LCMV-GP-derived epitope on MHC class II molecules but not a LCMV-NP derived epitope. In contrast, APCs from normal mice always presented both the GP-and NP-derived epitopes in comparable amounts. In consequence, LCMV-specific Th cells from Ii-deficient mice were found to exhibit exclusively LCMV-GP specificity in the initial
phase of the CD4+ T cell response. At later timepoints after infection also LCMV-NP-specific Th cells could be detected most probably due to CTL-mediated lysis of infected cells leading to locally high external antigen concentrations that over-ride the Ii-deficiency. In contrast, Th cell responses induced in normal mice upon LCMV infection exhibited similar LCMV-GP and LCMV-NP specificity at all timepoints. Thus, in vivo MHC class II loading by endogenously synthesized membrane LCMV-glycoprotein is functional and becomes dominant when the classical endosomal processing pathway is abrogated.
Materials and Methods

Mice
C57BL/6 mice were obtained from the breeding colony of the Institut für Zuchthygiene, Zürich. The generation of the mice deficient for the invariant chain has been described previously (Viville et al., 1993). Mice were bred in a conventional mouse house facility.

Viruses
The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube, Hamburg, Germany and grown on L929 cells with a low multiplicity of infection.

The recombinant baculoviruses expressing the nucleoprotein of the LCMV-virus have been described (Matsuura et al., 1987). Recombinant baculoviruses were derived from nuclear polyhedrosis virus and were grown at 28°C in Spodoptera frugiperda cells in TC-100 medium (Gibco Basel, Switzerland). Proteins were produced as described (Bachmann et al., 1994a).

Cell lines and T cell epitopes
The generation and analysis of the two T cell hybridomas 5A1 (LCMV-GP-specific) and VE8 (LCMV-NP-specific) has been described previously (Oxenius et al., 1995). By use of these hybridomas epitope mapping of the LCMV-GP and LCMV-NP-derived MHC class II binding peptides could be performed: The I-A^b-binding peptide P13 consists of amino acids 61-80 of the LCMV-GP and the I-A^b-binding peptide P61 consists of amino acids 309-328 of the LCMV-NP. The recombinant bacteria expressing the LCMV GP2 protein have been described previously (Weidt et al., 1994). Expression of the recombinant protein in E.coli cells was followed by purification via affinity chromatography as previously described (Weidt et al., 1994). Protein concentrations were determined by UV spectroscopy.

Epitope presentation by in vivo infected APCs
C57BL/6 or Ii-deficient mice were immunized with 2x10^4 pfu (or 200) pfu of LCMV and 2 to 4 days later spleen cells from LCMV-infected as well as from naive control mice were irradiated (2500 cGy) and incubated in 3-fold serial dilutions (highest concentration: 3x10^6 cells /well) together with 4x10^4 5A1 or VE8 T-cell hybridomas, respectively for 20 hrs. IL-2 concentrations in the supernatants were determined using the IL-2-sensitive cell line CTLL-2 followed by AlamarBlue™ (Biosource, International) colour reaction. In a second type of experiments, C57BL/6 or Ii-deficient mice were inoculated intraperitoneally with thioglycollate and 5 days later infected with 200 pfu LCMV
intraperitoneally. One day later peritoneal macrophages were harvested, irradiated (3000 cGy) and incubated in 3-fold serial dilutions (highest concentration: 1x10⁵ cells /well) without (negative control) or with 4x10⁴ 5A1 or VE8 T-cell hybridomas, respectively for 20 hrs. IL-2 concentration in the supernatants were determined as described above.

**T-cell proliferation**

C57BL/6 or Ii-deficient mice were immunized with 200 pfu of LCMV-WE intravenously. At daily intervals ranging from d5 to d12 after infection and additionally at d15 and d20 after infection, CD4⁺ T-cells were purified from spleen cell suspensions by MACS sorting according to the protocol of the supplier (Miltenyi Biotec, Bergisch Gladbach, Germany). 1x10⁵ CD4⁺ T cells were incubated in 96-wells with 3-fold serial dilutions of P13 or P61 (highest conc.: 5 μg/ml) or medium only in the presence of 7x10⁵ irradiated (2000 cGy) C57BL/6 spleen cells for 3 days. Proliferation was assessed by incorporation of ³H-thymidine (25 μCi/well). P13 and P61 represent I-Ab restricted T cell epitopes of the glycoprotein and nucleoprotein respectively of LCMV which have been described elsewhere (Oxenius et al., 1995).

**Cytokine analysis**

Supernatants of proliferation assays obtained as described above were analyzed for IFNγ content (72 hrs after restimulation). Supernatants were serially diluted in 3-fold steps and IFNγ was assessed by ELISA as described (Chirmule, 1991; Pruslin, 1991).

In brief, 96-well plates (Petra Plastik, Chur, Switzerland) were incubated overnight with anti-IFNγ antibody (0.2 μg/well; PharMingen, San Diego, CA) in 0.1M NaHCO₃, pH 8.2 at 4°C. Plates were then preincubated with 2% bovine serum albumine in phosphate-buffered saline for 2 h and washed, and serial dilutions of supernatants were added to the wells and incubated for 3 hrs. Plates were washed and incubated with a biotinylated anti-IFNγ anibody (0.1 μg/well; PharMingen, San Diego, CA) for 1 hr at room temperature. After washing peroxidase-conjugated streptavidin (50 ng/well; Jackson ImmunoResearch Laboratories, Inc.) was added. After 30 min, plates were washed and developed with ABTS (5 mg of 2,2'−azino-di-3-ethyl-benzthiazolinsulfonate and 20 μl of H₂O₂ in 50 ml of NaHCO₃ [pH4]). Optical densities were determined at 405 nm.

**ELISA**

The LCMV-specific enzyme-linked immunosorbent assay (ELISA) has been described previously (Battegay et al., 1993). 96-well plates (Petra Plastik, Chur, Switzerland) were incubated with recombinant LCMV-NP (10 ng/well) in 0.1M
NaH₂PO₄, pH 9.4 at 4°C. Plates were then preincubated with 2% bovine serum albumine in phosphate-buffered saline for 2 h and washed, and serial dilutions of serum samples (30-fold prediluted) were added to the wells and incubated for 90 min. Plates were washed and incubated with horse raddish peroxidase-labelled goat anti-mouse IgG (Sigma). After 90 min, plates were washed and developed with ABTS (5 mg of 2,2'-azino-di-3-ethyl-benzthiazolinsulfonate and 20 μl of H₂O₂ in 50 ml of NaHCO₃ [pH4]). Optical densities were determined at 405 nm.

LCMV-GP2-specific IgG titers were measured on ELISA-plates coated with 0.1 μg/well purified recombinant LCMV-GP2.

**LCMV replication in normal and Ii-deficient macrophages**

Thioglycollate-activated macrophages harvested from naive C57BL/6 mice or Ii-deficient mice were incubated in IMDM with 10% FCS and antibiotics overnight. Adherent macrophages were then infected with LCMV at a MOI of 1 and were harvested at three-hourly intervals (from 3 to 21 h after infection). 2x10⁵ harvested macrophages were centrifuged onto SuperFrost/Plus microscope slides (Menzel-Gläser, Germany) in a Cytospin-0030 (Shandon-Southern, Runcorn, England). Cytopreps were fixed by a 30 minute exposure to acetone before intracellular LCMV-NP protein was stained as described previously (Battegay et al., 1991).
Results

*Endogenously synthesized LCMV-GP is presented on LCMV-infected Ii-deficient APCs in vitro*

We have previously shown that endogenously synthesized LCMV-GP can load MHC class II molecules independently of endosomal processing and/or the presence or absence of Ii (Oxenius et al., 1995). Therefore, *in vitro* infection of Ii-deficient APCs (exhibiting strongly reduced MHC class II loading by exogenous soluble proteins (Viville et al., 1993)) with LCMV leads to presentation of a LCMV GP-derived epitope (peptide P13) (Fig.1A) but not to presentation of a LCMV NP-derived epitope (peptide P61) (Fig.1B). In contrast, *in vitro* infection of normal APCs with LCMV leads to MHC class II presentation of both the GP- and NP-derived epitopes (Fig.1C+D). MHC class II presentation of these two epitopes was analyzed by means of activation of two T cell hybridomas specific for these epitopes which results in IL-2 production. Since both hybridomas show comparable antigen-sensitivities, the MHC class II loading observed in Ii-deficient APCs was not due to a higher sensitivity of the LCMV-GP-specific hybridoma 5A1 (not shown, (Oxenius et al., 1995)). In addition, LCMV GP synthesis is initiated only after LCMV NP synthesis in LCMV-infected cells and LCMV GP is also quantitatively less abundant (Buchmeier et al., 1978; Vezza et al., 1977); this excludes the possibility that MHC class II presentation of endogenously synthesized LCMV GP is due to higher intracellular GP concentrations as compared to NP concentrations.
Fig. 1 In vitro LCMV-infected Ii-deficient APCs exclusively present endogenously synthesized LCMV-GP. Spleen cells from Ii-deficient mice (panels A+B) and normal control mice (panels C+D) were infected in vitro with LCMV and MHC class II presentation of LCMV-derived epitopes was monitored by activation-induced IL-2 secretion by either the LCMV-GP-specific T cell hybridoma 5A1 (squares, left graph) or the LCMV-NP-specific T cell hybridoma VE8 (squares, right graph). As controls for activation Ii-deficient and normal APCs were pulsed with the appropriate peptides recognized by the hybridomas: with LCMV-GP-derived P13 (circles) and with LCMV-NP-derived peptide P61 (triangles). One of three comparable experiments is shown.
In order to formally prove that LCMV-NP is present and that its synthesis is initiated at the same timepoint in comparable levels in LCMV-infected C57BL/6 and Ii-deficient cells, the following experiment was performed. Peritoneal macrophages from either normal or Ii-deficient mice were infected \textit{in vitro} with LCMV. In 3 hourly intervals - from 3 hrs to 21 hrs after infection - macrophages were harvested and intracellularly synthesized LCMV-NP was visualized by staining with the NP-specific monoclonal antibody VL4 (Battegay et al., 1991). As summarized in table I, significant LCMV-NP staining was observed in both normal and Ii-deficient macrophages 15 hrs after initial infection, demonstrating that a lacking invariant chain does not influence LCMV-NP synthesis.

Table I: Assessment of LCMV-NP in LCMV-infected macrophages from normal C57BL/6 and Ii-deficient mice. Peritoneal macrophages were infected in vitro with LCMV and intracellular presence of LCMV-NP was analyzed by staining with the NP-specific monoclonal antibody VL4 in 3 hr intervals after initial infection.

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<td>Ii-/- macrophages</td>
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neg.: LCMV-NP not detectable
+, ++, +++: VL-4 stained cells detected at different frequencies

In addition, LCMV-titers in spleens of control or Ii-deficient mice were determined three, four and five days after LCMV infection. C57BL/6 and Ii-deficient spleens showed comparable viral titers (data not shown). Thus, LCMV replication \textit{in vivo} exhibited indistinguishable kinetics in control and Ii-deficient mice.
Endogenously synthesized LCMV-GP is presented in Ii-deficient APCs in vivo upon LCMV infection

Alternative MHC class II loading pathways are in general technically difficult to assess in the presence of functional MHC class II loading/processing pathways. However, if they are nonfunctional or functional at significantly reduced levels, alternative MHC class II loading pathways may be observable.

We addressed the question whether MHC class II loading by endogenously synthesized LCMV-GP can be observed in vivo in an environment where classical MHC class II presentation pathways are severely hampered. We therefore used Ii-deficient and normal control mice and infected them with LCMV. Two or four days after infection spleen cells were harvested, irradiated and used as APCs for activation of the two T cell hybridomas 5A1 and VE8 (Fig.2). Whereas both LCMV-GP- and LCMV-NP-derived epitopes were presented on MHC class II molecules from normal LCMV-infected control mice (Fig.2A+C), only the LCMV-GP-derived epitope PI3 was detectable on APCs from LCMV-infected Ii-deficient mice (Fig.2B+D). These results demonstrate - in analogy to the in vitro results - that upon in vivo infection of Ii-deficient mice with LCMV only endogenously synthesized membrane GP but not cytosolic NP can be presented efficiently on MHC class II molecules.
Fig. 2 *In vivo* MHC class II presentation of LCMV-derived epitopes on APCs upon LCMV infection of normal or Ii-deficient mice. Two days (panels A+B) or four days (panels C+D) after intravenous infection of control (panels A+C) or Ii-deficient mice (panels B+D) with LCMV. Spleen cells were analysed for MHC class II associated presentation of the two LCMV-derived epitopes P13 (GP) (filled circles) and P61 (NP) (open squares) by determining activation-induced secretion of IL-2 by the hybridomas 5A1 or VE8. As negative controls spleen cells from naive control (open triangles) or Ii-deficient mice (open diamonds) were included in the experiment. One of three comparable experiments is shown.
In a second attempt we focussed on MHC class II loading by endogenously synthesized LCMV-GP \textit{in vivo} in a more defined APC population. It is known that macrophages are readily infected by LCMV \textit{in vivo} (Buchmeier et al., 1980) and we therefore used \textit{in vivo} infected peritoneal macrophages to analyze MHC class II presentation of LCMV-derived epitopes. Ii-deficient and normal mice were infected intraperitoneally with LCMV and 1 day later the peritoneal macrophages were harvested, irradiated and used as APCs for activation of the GP- and NP-specific hybridomas (Fig.3). Whereas \textit{in vivo} infected macrophages from normal control mice presented both the GP- and NP-derived epitopes with comparable efficiency (Fig.3A), macrophages originating from LCMV-infected Ii-deficient mice only presented the GP-derived epitope in a functional fashion. (Fig.3B).

![Graph](image)

Fig.3 \textit{In vivo} MHC class II presentation of LCMV-derived epitopes on peritoneal macrophages upon LCMV infection of normal or Ii-deficient mice. Thioglycollate-activated macrophages in control (left graph) or Ii-deficient mice (right graph) were infected in vivo by intraperitoneal infection with LCMV. One day later macrophages were harvested and MHC class II associated presentation of the two LCMV-derived epitopes P13 (GP) and P61 (NP) was assayed by determining activation-induced secretion of IL-2 by the hybridomas 5A1 (filled circles) or VE8 (open squares). In addition, macrophages from uninfected control mice (open triangles, left graph) or uninfected Ii-deficient mice (open diamonds, right graph) were incubated in the presence of T cell hybridomas. One of three comparable experiments is shown.
Thus, in two independent assays it was demonstrated that endogenously synthesized LCMV-GP but not endogenously synthesized LCMV-NP was able to load MHC class II molecules in Ii-deficient mice in vivo after viral infection.

**Kinetics of induction of Th cells exhibiting GP- or NP-specificity**

The preferential MHC class II presentation of the GP-epitope derived from endogenously synthesized LCMV-GP in APCs from Ii-deficient mice as compared to APCs from control mice which were able to comparably stimulate the GP- and NP-specific T cell hybridomas may have a direct influence on the induction of Th cells exhibiting either GP- or NP-specificity. To assess this question, proliferation experiments using purified CD4⁺ T cells from either LCMV-infected Ii-deficient or normal mice were performed. The time course of the induction of Th cells having either GP- or NP-specificity was followed by a recall proliferation assay with spleen cells taken in daily intervals after LCMV-infection, from day 5 to day 10 after infection; in addition, two later timepoints after infection (d15 and d20) were included in the experiment. CD4⁺ T cells were purified from spleen cell suspensions of LCMV-infected Ii-deficient or normal mice and LCMV-specific proliferation was determined using as LCMV-specific antigens either the GP- or NP-derived peptides (P13 and P61) presented on irradiated C57BL/6 spleen cells as ACPs (Fig.4). In general, fewer antigen dilution steps could be performed in proliferation experiments with Th cells from Ii-deficient mice due to the reduced CD4⁺ T cell numbers in Ii-deficient mice (Bikoff et al., 1995; Tourne et al., 1995; Viville et al., 1993). In Fig.4, data from d6, d8, d10 and d15 after LCMV infection are shown. T cell specificities on d15 were similar to those on day 20 after infection (not shown). Th cells from LCMV-infected control mice (Fig.4, upper row) exhibited both GP- and NP specificities in a time-correlated fashion. In contrast, Th cells originating from LCMV-infected Ii-deficient mice were exclusively specific for the GP-derived peptide P13 in between d7 and d10 after LCMV infection (Fig.4, lower row). This correlated with the observed LCMV-GP confined MHC class II presentation on d2 and d4 after infection (Fig.2). However, the situation was different on d15 and later after infection: Th cells from LCMV-infected Ii-deficient mice started to exhibit both GP- and NP-specificities. This late induction of Th cells having NP-specificity in Ii-deficient mice might be attributed to the strong CTL-response elicited after LCMV infection which causes massive lysis of LCMV-infected cells thereby liberating LCMV-antigens in high local concentrations which might then load MHC class II molecules on neighbouring APCs by-passing the Ii-deficiency.
Fig. 4 Kinetics of LCMV-GP or LCMV-NP-specific proliferative responses in control or Ii-deficient mice. C57BL/6 and Ii-deficient mice were immunized intravenously with 200 pfu LCMV and 6, 8, 10 and 15 days after infection CD4+ T cells were purified from spleen cell suspensions and restimulated with titrated amounts of peptide P13 (GP) (squares) or peptide P61 (NP) (circles) or without antigen (triangles) in the presence of irradiated C57BL/6 spleen cells. The upper row shows the 3H-thymidine incorporation results with CD4+ T cells from LCMV-immunized C57BL/6 mice whereas the lower row shows the results with CD4+ T cells from LCMV-immunized Ii-deficient mice. Data points represent 3-fold dilutions of antigen; dilution curves all start at the same antigen concentration. One of two comparable experiments is shown.
In parallel, IFNγ secretion by in vitro restimulated LCMV-specific Th cells was analyzed in the same time-course experiments. Supernatants of the proliferation assays shown in Fig.4 were collected 72 hrs after restimulation, were serially diluted and IFNγ contents were determined by ELISA (Fig.5). Note that the sometimes high background for IFNγ production does not reflect technical difficulties in IFNγ assessment but is due to IFNγ production by \textit{in vivo} activated Th cells in the absence of exogenously added antigen. The first and third row show the IFNγ secretion of Th cells from LCMV-infected control mice whereas the second and fourth row show IFNγ secretion of Th cells from LCMV-infected Ii-deficient mice. Similar to the proliferation results, Th cells from control mice secreted comparable amounts of IFNγ after restimulation with either the NP- or GP-derived peptide. In contrast, Th cells from LCMV-infected Ii-deficient mice only secreted significant amounts of IFNγ upon restimulation with the GP-derived peptide P13 if analyzed between d7 and d10 after infection. At later timepoints - as shown on d15 after infection - both restimulation with the GP- or NP- derived peptides induced IFNγ secretion.
Fig. 5 Kinetics of LCMV-GP or LCMV-NP-specific IFNγ secretion in CD4+ T cells from control or Ii-deficient mice. Supernatants from the proliferation assays shown in Fig. 4 were collected 72 hrs after restimulation, were serially diluted and analyzed for IFNγ content by ELISA. CD4+ T cells from LCMV-immunized control mice (first and third row) or LCMV-immunized Ii-deficient mice (second and fourth row) were restimulated with peptide P13 (squares) or peptide P61 (circles) or without antigen (triangles) in the presence of irradiated C57BL/6 spleen cells. Data points represent 3-fold dilutions of cell culture supernatant; dilution curves all start with neat supernatant. One of two comparable experiments is shown.
To address the possibility that the delayed NP-specific Th cell response is due to a defect in the T cell repertoire in the invariant chain-deficient mice rather than due to the inability to present endogenously synthesized NP, Ii-deficient mice and normal C57BL/6 mice were infected intravenously with a high dose of LCMV (2x10^6 pfu as compared to 200 pfu in the former experiments), thus increasing the initial NP-antigen dose already by a factor of 10000. 10 days after infection, CD4^+ T cells were purified from spleen cell suspensions and recall proliferations to the NP-derived peptide P61 were performed (Fig.6). Both CD4^+ T cell populations from Ii-deficient mice and from control mice mounted a significant proliferative response against the peptide P61 already 10 days after infection with a high dose of LCMV. This supports the notion that the delayed NP-specific proliferative response in Ii-deficient mice after low dose LCMV infection is rather due to an impaired MHC class II antigen presentation which can partially be overcome by increasing the antigen load (Battegay et al., 1996) in the case of high dose LCMV infection and not due to a NP-specific defect in the T cell repertoire.

Fig.6 LCMV NP-specific proliferative response of CD4^+ T cells from Ii-deficient or normal control mice after high dose LCMV infection. Ii-deficient (left panel) and normal control mice (right panel) were infected with 2x10^6 pfu LCMV intravenously and 10 days later splenic CD4^+ cells were restimulated with graded amounts of the LCMV NP-derived peptide P61 (circles) or with no antigen (diamonds) in the presence of irradiated C57BL/6 spleen cells. Data points represent 3-fold dilutions of peptide antigen; dilution curves all start at the same antigen concentration. One of two comparable experiments is shown.
Taken together, measurement of two parameters - i.e. antigen-dependent proliferation and IFNγ secretion - indicative of Th cell induction *in vivo* demonstrated that LCMV-GP-specific Th cells are induced significantly earlier in Ii-deficient mice upon LCMV infection as compared to LCMV-NP-specific Th cells whereas both specificities are observed with equal induction kinetics in normal control mice.

*LCMV-specific antibody response in Ii-deficient mice*

LCMV-specific antibody responses are T-dependent and are therefore another - albeit indirect - parameter for measuring functional T help *in vivo*. We analyzed ELISA-binding LCMV NP-specific IgG responses in Ii-deficient and normal mice upon i.v. infection with 200 pfu LCMV. Although kinetics of LCMV NP-specific IgG antibodies were shifted to later timepoints in Ii-deficient mice as compared to control mice, significant titers were reached within twelve days after infection (Fig. 7A). As expected, no LCMV NP-specific antibody response could be observed in Ii-deficient mice if they were immunized with non-replicating, UV-inactivated LCMV in CFA subcutaneously (Fig. 7B) in contrast to control mice which generated a strong LCMV NP-specific IgG response by 9 days after immunization.
Fig. 7 LCMV NP-specific IgG response in Ii-deficient mice upon LCMV infection or upon immunization with non-replicating, UV-inactivated LCMV. Fig.7A: Ii-deficient mice (circles) and normal C57BL/6 control mice (squares) were immunized with 200 pfu LCMV intravenously and sera were collected at the indicated timepoints. In addition normal C57BL/6 serum was included as negative control (triangles). LCMV NP-specific IgG antibodies were determined by ELISA in 30-fold prediluted sera. Each line represents one individual mouse. Fig.7B: Ii-deficient mice (circles) and normal control mice (squares) were immunized subcutaneously with UV-inactivated LCMV which was emulsified in CFA or with CFA alone (triangles). LCMV NP-specific IgG titers were determined at the indicated timepoints after immunization in 30-fold prediluted sera. Each line represents one individual mouse. One of three comparable experiments is shown.
Thus, LCMV NP-specific IgG responses are only induced in Ii-deficient mice if infectious LCMV is used for immunization but not if a nonreplicating form of the virus in adjuvant was used. Interestingly, analysis of LCMV GP2-binding IgG antibody responses in comparison to LCMV NP-binding IgG antibody responses in Ii-deficient and control mice revealed, that 10 days after infection with either low dose (Fig.8A) or high dose LCMV (Fig.8C), LCMV GP2-binding antibodies were equally induced in Ii-deficient and control mice. This suggests that the early induced LCMV-GP-specific Th cells in Ii-deficient mice were comparable to normal mice able to mediate help for LCMV-GP2-specific B cells. In contrast, LCMV NP-binding antibody titers were strongly reduced in Ii-deficient mice 10 days after low dose LCMV infection (Fig.8B) as compared to control mice. However, high dose LCMV infection significantly increased the LCMV NP-specific IgG response in Ii-deficient mice (Fig.8D), suggesting that this increase in NP-specific IgG response is due to an enhanced NP-specific Th cell response in the high dose infected Ii-deficient mice, which was apparently induced by an increased antigen load (Battegay et al., 1996).
Fig. 8 LCMV GP2-specific and LCMV NP-specific IgG responses in Ii-deficient and control mice after low or high dose infection with LCMV. Ii-deficient or control mice were infected intravenously with low dose LCMV and LCMV GP2-binding (A) or LCMV NP-binding (B) IgG titers were determined 10 days after infection. In addition, Ii-deficient and control mice were infected intravenously with a high dose of LCMV and LCMV GP2-binding (C) or LCMV NP-binding (D) IgG titers were determined 10 days after infection. IgG titers were assessed in 30-fold prediluted sera and each line represents one individual mouse. One of two comparable experiments is shown.
Discussion

This study reports on direct in vivo MHC class II loading by the endogenously synthesized LCMV membrane glycoprotein upon infection of Ii-deficient mice with LCMV. In contrast, endogenously synthesized cytosolic LCMV-NP can not be loaded onto MHC class II molecules in vivo in an invariant chain-deficient environment early after infection most likely because this process is dependent on action of CD8+ effector CTLs. Analysis of MHC class II presentation of LCMV-derived epitopes was restricted to the two epitopes P13 and P61 recognized by the T cell hybridomas 5A1 and VE8, respectively, since these two peptides are the only LCMV-GP- and LCMV-NP-derived epitopes presented by the I-A^b molecule.

The in vivo findings correlate with previously reported in vitro results where it has been shown, that endogenously synthesized LCMV-GP but not endogenously synthesized LCMV-NP was able to gain access to MHC class II molecules in a chloroquine- and leupeptine- insensitive manner as well as independently of the presence of the invariant chain (Oxenius et al., 1995).

The ability of endogenously synthesized LCMV-GP to efficiently associate with MHC class II molecules in the absence of a functional invariant chain was reflected in the specificity patterns of Th cells induced in Ii-deficient mice upon infection with LCMV. LCMV-specific Th cell responses induced in Ii-deficient mice could be divided into two phases: In an earlier phase (day 7 to day 10 after LCMV infection), Th cells exhibited exclusively LCMV-GP specificity - as could be expected from the fact, that only the GP-derived peptide is measurably presented on MHC class II molecules on Ii-deficient APCs early after infection. During a second phase after infection (later than day 10), also NP-specific Th cells were induced in Ii-deficient mice. This late induction of NP-specific Th cells may be either explained by the impaired MHC class II presentation of LCMV NP in Ii-deficient mice or by the fact that Ii-deficient mice exhibit a CD4+ T cell compartment which is reduced in size and might also be altered in its repertoire of specificities. To test for this second hypothesis, Ii-deficient mice were infected with a high dose of LCMV, thus drastically increasing the initial antigen load. In this situation, NP-specific Th cell proliferation was measurable already 10 days after infection, indicating that the delayed induction of NP-specific Th cells after low dose LCMV infection of Ii-deficient mice was not due to a defect in the NP-specific Th cell repertoire but rather due to an impaired antigen presentation of the NP-derived Th cell epitope at early timepoints after low dose infection where antigen concentrations are limiting for Ii-deficient APCs.

This view is supported if the viral characteristics of the LCMV infection in vivo are taken into account: LCMV is a non-cytopathic virus and therefore replicates in host cells without lysing them and releasing viral antigens (Buchmeier et al., 1980;
Lehmann, 1971). This implies that viral proteins which can be taken up by APCs via endocytosis are present in low amounts at early timepoints after infection. These low amounts of viral antigens are sufficient under normal circumstances (functional antigen presenting capacity of APCs) to efficiently load MHC class II molecules and thereafter to activate Th cells. Yet, under critically impaired MHC class II loading conditions -as they exist in invariant chain-deficient mice- these low exogenous antigen levels are not sufficient to prime T helper cells. LCMV infection elicits a vigorous CTL response in normal and also in Ii-deficient mice (Battegay et al., 1996) which causes massive lysis of LCMV-infected cells and leads to immunopathology by day 7-9 after infection. By the CTL-mediated lysis of LCMV-infected cells, substantial amounts of cell-associated virus antigen will be locally released (Kyburz et al., 1993) which seem sufficiently concentrated to locally overcome the Ii-deficiency and load MHC class II molecules of recruited APCs. In fact it has been shown that very high concentrations of different soluble protein antigens lead to efficient MHC class II loading in Ii-deficient APCs in vitro (Viville et al., 1993) and in vivo (Battegay et al., 1996). It might therefore be expected that at the timepoint of maximal CTL activity which peaks around d7-9 after infection, also LCMV-NP-antigen in native or degraded form becomes sufficiently abundant to load MHC class II molecules and to induce measurable NP-specific Th cell proliferation by day 15 after infection. Attempts at detecting LCMV-derived epitopes on MHC class II molecules of APCs taken ex vivo on d8 after LCMV-infection failed. This was probably due to the limited numbers of functional APCs in immunopathologically damaged spleen tissue; in addition a critical density of APCs that present certain limiting antigens might be reached in vivo but the necessary conditions may not be fulfilled after disruption of the spleen architecture and transfer of single cells to plastic culture dishes.

The assumption that CTL-mediated lysis of LCMV-infected cells is locally liberating sufficiently concentrated antigen concentrations to over-ride the Ii-deficiency has been impossible to formally prove since in vivo viral kinetics are critically controlled by LCMV-specific CTLs. Thus, in vivo depletion of CD8+ T cells results in uncontrolled virus replication which rapidly leads to very high antigen concentrations apparently reaching the critical threshold for MHC class II loading in the absence of the invariant chain. Indeed, infection of Ii-deficient mice with a high dose of LCMV induced NP-specific Th cell responses with enhanced kinetics as compared to low dose infection, thus further strengthening the notion that high antigen concentrations lead to MHC class II presentation of antigenic epitopes in the absence of the invariant chain.

The kinetics of LCMV NP-specific IgG responses are delayed after LCMV infection of Ii-deficient mice as compared to normal mice. However, enhanced LCMV NP-
specific IgG responses were observed in Ii-deficient mice after infection with a high
dose of LCMV. Since the virus internal LCMV nucleoprotein is most probably
recognized by NP-specific B cells in a form where it is no longer physically
associated with the LCMV GP, one would assume that only NP-specific Th cells are
able to mediate help for NP-specific B cells (intramolecular help). Infection of li-
deficient mice with high doses of LCMV leads to an earlier induction of NP-specific
Th cells as compared to low dose LCMV infection which would explain the enhanced
NP-specific IgG response observed after high dose LCMV infection. Low dose
LCMV infection of normal C57BL/6 mice, on the other hand, readily induced a NP-
specific Th cell response which is reflected by the efficient NP-specific IgG response.
Interestingly, LCMV GP2-binding IgG responses after low or high dose LCMV
infection of li-deficient mice were identical to those from normal control mice.
Apparently, the LCMV GP-specific Th cells which are readily induced upon LCMV
infection of li-deficient mice are able to provide help for LCMV GP2-specific B cells
but not for LCMV NP-specific B cells. Thus, the identical LCMV GP2-specific IgG
response observed in li-deficient and normal mice upon LCMV infection further
strengthens the finding that LCMV GP-specific Th cells are induced with enhanced
kinetics - due to MHC class II loading by intracellularly synthesized LCMV GP - as
compared to LCMV NP-specific Th cells in li-deficient mice.
Immunization of li-deficient mice with non-replicating antigens such as UV-
inactivated LCMV inoculated subcutaneously in CFA does not result in any
measurable LCMV-specific antibody response, suggesting that exogenous local
antigen concentrations in absence of infection are not sufficient for reaching the
critical threshold to overcome the li-deficiency.
Analysis of virus-specific antibody responses in li-deficient mice after infection with
vesicular stomatitis virus (VSV) revealed that li-deficient mice mounted a
surprisingly efficient IgG responses against the glycoprotein of VSV (Battegay et al.,
1996). Since VSV viral particles -being type I T-independent B cell antigens- induce
an extremely efficient B cell response due to the highly organized glycoprotein in
the viral envelope (Bachmann et al., 1993), this model antigen needs little T help for
the switch to IgG (Bachmann and Kündig, 1994). This is in contrast to LCMV
infection where virus-specific B cell activation, IgM and IgG production are all
critically dependent on T help. In addition, VSV is a cytolytic virus which
presumably rapidly leads to high local antigen concentrations at early time points
after infection (Battegay et al., 1996). Whether the efficient VSV G-specific IgG
response upon infection of li-deficient mice is due to MHC class II presentation of
endogenously synthesized VSV G or a consequence of cell destruction by VSV that
leads to high local antigen concentrations remains to be answered and probably both
are involved in the generation of an efficient VSV G specific IgG response.
The findings that VSV infection of Ii-deficient mice induced a virus-neutralizing IgG response with normal kinetics and that LCMV infection leads to an efficient LCMV GP2-specific IgG response indicate that the B cell compartment of Ii-deficient mice is not functionally impaired and thus not responsible for the delayed kinetics of LCMV NP-specific responses in Ii-deficient mice. This is corroborated by the observation that LCMV NP-specific IgG responses are induced with enhanced kinetics after high dose LCMV infection of Ii-deficient mice which favours the notion that MHC class II-associated antigen presentation constraints of LCMV NP are responsible for the delayed NP-specific IgG response observed after LCMV infection of Ii-deficient mice. These studies of B cell responses to viral antigens in Ii-deficient mice are in contrast to a recent report describing a B cell maturation defect in Ii-deficient mice (Shachar and Flavell, 1996).

Taken together, in vivo MHC class II loading by endogenously synthesized membrane-associated LCMV-GP but not by endogenously synthesized cytosolic LCMV-NP was observed in LCMV-infected mice. This MHC class II loading by endogenously synthesized LCMV-GP is functional in vivo by inducing exclusively LCMV-GP-specific Th cells in a first phase of Th cell induction in an in vivo setting exhibiting an impaired MHC class II antigen presentation capacity. These results suggest that such an additional MHC class II loading pathway used by certain endogenously synthesized proteins may be important early after infection - particularly for non-cytopathic viruses which infect MHC class II positive cells - because this may critically enhance the generation of a protective antibody response.
Chapter 3:

**CD40-CD40 Ligand interactions are critical in T-B cooperation but not for other anti-viral CD4+ T cell functions**

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Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; VSV, vesicular stomatitis virus; pfu, plaque-forming unit; DNP, dinitrophenol; VaccGI ND, recombinant vaccinia virus expressing VSV glycoprotein; KLH, keyhole limpet hemocyanine; OVA, ovalbumin; HEL, hen egg lysozyme; LCMV-DNP, DNP covalently coupled to LCMV.
Abstract. CD40-CD40 ligand (CD40L) interaction is required for the generation of antibody responses to T-dependent antigens as well as for the development of germinal centers and memory B-cells. The role of the CD40-CD40L interaction in the induction of antigen-specific Th cells and in mediating Th cell effector functions other than cognate help for B cells is less well understood. Using CD40- and CD40L-deficient mice together with lymphocytic choriomeningitis virus and vesicular stomatitis virus as viral model antigens, this study corroborates earlier findings that no Ig isotype switching of virus-specific antibodies was measurable upon infection of CD40- or CD40L-deficient mice. In contrast, in vivo induction of virus-specific CD4+ T cells measured by proliferation and cytokine secretion of primed virus-specific Th cells in vitro was not crucially dependent on the CD40-CD40L interaction. In addition, virus-specific Th cells primed in a CD40-deficient environment, adoptively transferred into CD40-competent recipients, were able to mediate Ig isotype switch. Th-mediated effector functions distinct from and in addition to T-B collaboration were analyzed in CD40- and CD40L-deficient and normal mice: i) local inflammatory reactions upon LCMV infection mediated by LCMV-specific Th cells were not dependent on a functional CD40-CD40L interaction, ii) cytokine-mediated protection by CD4+ T cells primed by vesicular stomatitis virus against a challenge infection with recombinant vaccinia virus expressing the glycoprotein of vesicular stomatitis virus was found to be equivalent in CD40L-deficient and normal mice.

Thus, CD40-CD40L interaction plays a crucial role in T-B interactions for Th-dependent activation of B-cells but not, or to a much lesser extent, in T cell-antigen presenting cell interactions, antigen-specific Th cell responses in vitro and for interleukin-mediated Th cell effector functions in vivo.
Introduction

It has been established both in vitro and in vivo that the interaction between CD40 on B-cells and its ligand CD40L, which is expressed on activated Th cells, is required for Ig isotype switching, Ig-production and the formation of germinal centers (Banchereau et al., 1994; Foy et al., 1994; Foy et al., 1993; Lederman et al., 1992a; Lederman et al., 1992b; Noelle et al., 1992; Parker, 1993; Van den Eertwegh et al., 1993). In vivo, administration of anti-CD40L antibody or soluble CD40L (Gray et al., 1994; Lane et al., 1993) or CD40-Ig fusion protein (Noelle et al., 1992) as well as studies in CD40- or CD40L- deficient mice (Kawabe et al., 1994; Renshaw et al., 1994; Xu et al., 1994) have shown that the generation of primary and secondary humoral immune responses and the formation of germinal centers to a variety of thymus-dependent antigens were abrogated. Furthermore, patients with hyper-IgM (HIGM) syndrome, a genetic disorder due to mutations in the CD40L gene, exhibit an inability to respond to thymus-dependent antigens and have secondary lymphoid organs which are devoid of germinal centers. Nevertheless, patients suffering from HIGM have normal T cell numbers and are generally not more susceptible to viral infections than healthy individuals (Allen et al., 1993; Aruffo et al., 1993; Banchereau et al., 1994; Disanto et al., 1993; Farrington et al., 1994; Korthauer et al., 1993). This may be due to the presence of IgM antibodies and/or CD40L-independent T cell functions.

Since CD40 is not expressed solely on B-cells but also on dendritic cells, follicular dendritic cells, monocytes, hematopoietic progenitor cells and epithelial cells (Clark et al., 1992; Freudenthal and Steinmann, 1990; Galy and Spits, 1992; Ling et al., 1987; Schriefer et al., 1989) CD40-CD40L interaction might be important for the induction and/or effector phase of Th cells in general. This question was addressed in this study. We confirmed here in two infectious virus disease models that a functional CD40-CD40L interaction is not required for T help-independent anti-viral IgM responses but is strictly required for T-dependent Ig class switching of virus specific B cells. This study additionally investigated the importance of the CD40-CD40L interaction for the induction of Th cell responses and for Th cell effector functions other than cognate T help for B cells.

Using either CD40- or CD40L-deficient mice, Th cell mediated antiviral immune responses upon infection with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV) were investigated. We found that activation and proliferation of virus-specific Th cells did not require a functional CD40-CD40L interaction. Effector functions of Th cells were characterized by i) the analysis of antiviral humoral immune responses, ii) Th cell mediated inflammatory reaction upon LCMV infection and iii) Th cell mediated antiviral protection against vaccinia virus. Whereas IgM to IgG switch was completely abrogated in both CD40- and CD40L-
deficient mice, the two latter effector functions were comparable in CD40-, CD40L-deficient and normal mice, demonstrating a dichotomy in the role of CD40-CD40L interaction in Th cell effector functions.
Materials and Methods

Mice

Inbred C57BL/6 (H-2\textsuperscript{b}) mice were obtained from the breeding colony of the Institut für Zuchthygiene, Tierspital Zürich, Switzerland. The generation of mice deficient for CD40, CD40L or IgM expression has been described previously (Kawabe et al., 1994; Kitamura et al., 1992; Renshaw et al., 1994). Mice were bred in a conventional mouse house facility.

Viruses

The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube, Hamburg, Germany and grown on L929 cells (ATCC CRL 1) with a low multiplicity of infection.

VSV indiana (Mudd-Summers isolate) seeds, originally obtained from D. Kolakofsky, University of Geneva, were grown on BHK-21 (ATCC CRL 8544) cells infected at low multiplicity and plaqued on Vero cells.

Vaccinia virus expressing the glycoprotein of VSV was a generous gift of Dr. B. Moss (Laboratory of Viral Diseases, National Institutes of Health, Bethesda) (Mackett et al., 1985). Recombinant viruses were grown at low multiplicity of infection on BSC cells and plaqued on BSC cells.

The recombinant baculovirus expressing the LCMV nucleoprotein has been previously described (Battegay et al., 1993). The recombinant baculovirus was derived from nuclear polyhedrosis virus and was grown at 28°C in Spodoptera frugiperda cells in spinner cultures in TC-100 medium. Recombinant proteins were produced as previously described (Matsuura et al., 1987).

T cell proliferation

Mice were immunized intravenously with 200 pfu LCMV-WE. 14 days later, CD4\textsuperscript{+} T cells were purified from spleen cell suspensions by MACS-sorting according to the protocol of the supplier (Miltenyi Biotec, Germany). 1x10\textsuperscript{5} CD4\textsuperscript{+} T cells were incubated in 96-wells with 3-fold serial dilutions of either purified, UV-inactivated LCMV (concentrations shown for CD40L-/-, CD40-/-, IgM-/- and C57BL/6 ranged between 0.1 μg/ml and 1μg/ml), P13 (concentrations shown for CD40L-/-, CD40-/-, IgM-/- and C57BL/6 ranged between 0.1 μg/ml and 1μg/ml) or medium only in the presence of 7x10\textsuperscript{5} irradiated (2000cGy) C57BL/6 spleen cells for 3 days. Proliferation was assessed by incorporation of \textsuperscript{3}H-thymidine (25μCi/well). P13 represents an I-A\textsuperscript{b} restricted T cell epitope of the glycoprotein of LCMV which has been described elsewhere (Oxenius et al., 1995). We have previously shown that P13 is presented in vivo during a primary immune response against LCMV and that P13-specific CD4\textsuperscript{+} T cells are induced in vivo (Oxenius et al., 1995).
Cytokine analysis
Supernatants of proliferation assays as described above were analyzed for IL-2 content (24 hrs after restimulation), IFNγ content (60 hrs after restimulation) and IL-4 content (60 hrs after restimulation). IL-2 was determined using the IL-2-dependent cell line CTLL-2. Quantification of viable cells was performed by AlamarBlue™ colour reaction (Biosource, International) and measured by fluorescence emission at 590 nm using the CytoFluor™ 2350 (Millipore) fluorimeter. IFNγ and IL-4 were assessed by ELISA as described (Chirmule, 1991; Pruslin, 1991).

Adoptive transfer of primed Th cells followed by challenge with DNP-modified LCMV
CD40-deficient and CD40 competent mice as well as IgM-deficient mice were immunized with 200 pfu of LCMV-WE into both hind foot pads. 18 days later, single cell suspensions were prepared from spleens. 6x10^7 primed spleen cells or an equivalent number of naive cells were transferred intravenously into naive, sex-matched C57BL/6 recipients. Donor cells were pooled from two to three individuals. A few hours later mice were challenged intravenously with 0.4 µg of purified LCMV to which DNP has been covalently coupled (LCMV-DNP) (Mishell and Shiigi, 1980). Mice were bled 9 days later and DNP-specific IgG titers were determined by ELISA. The optimal dose for the challenge immunization with LCMV-DNP was determined by in vivo titration of the LCMV-DNP stock in naive versus LCMV primed mice. The optimal dose was chosen such that only LCMV primed mice but not naive mice gave rise to a DNP-specific IgG titer.

In addition, the same adoptive transfer experiment was performed using purified, LCMV-primed CD4+ T cells for transfer. CD40-deficient and heterozygous littermates were immunized with 200 pfu LCMV i.v.. 14 days later, CD4+ T cells were purified from spleen cell suspensions by MACS-sorting according to the protocol of the supplier (Miltenyi Biotec, Germany) and were used at a purity of at least 95 % as checked by FACS analysis. 6x10^6 purified T cells were adoptively transferred into naive C57BL/6 recipients and challenged with 0.4 µg LCMV-DNP. Whole spleen cell transfer from LCMV-primed CD40-deficient mice served as positive control. Mice were bled 10 days later and DNP-specific IgG titers were determined by ELISA.

ELISA
The LCMV nucleoprotein-specific enzyme-linked immunosorbent assay (ELISA) has been described previously (Battegay et al., 1993). 96-well plates (Petra Plastik, Chur, Switzerland) were incubated with LCMV nucleoprotein (0.01 µg/well) in 0.1M NaH₂PO₄, pH 9.4 at 4°C. Plates were then preincubated with 2% bovine serum
albumine in phosphate-buffered saline for 2 h and washed, and serial dilutions of serum samples (30-fold prediluted) were added to the wells and incubated for 90 min. Plates were washed and incubated with horse raddish peroxidase-labelled goat anti-mouse IgG (Sigma). After 90 min, plates were washed and developed with ABTS (5 mg of 2,2’-azino-di-3-ethyl-benzthiazolinsulfonate and 20 µl of H2O2 in 50 ml of NaHCO3 [pH4]). Optical densities were determined at 405 nm.

ELISA-measurement of DNP-specific IgG titers was performed similarly. ELISA-plates were coated with 0.25 µg/well of Ovalbumin covalently coupled to DNP (Charan et al., 1986; Mishell and Shiigi, 1980).

**Serum neutralization test**
Neutralizing titers of sera were determined as described (Roost et al., 1990). Sera were prediluted 40-fold in supplemented MEM and heat-inactivated for 30 min at 56°C. Serial twofold dilutions were mixed with equal volumes of virus diluted to contain 500 pfu/ml. The mixture was incubated for 90 min at 37°C in an atmosphere containing 5% CO2. One hundred µl of the serum-virus mixture were transferred onto Vero cell monolayers in 96-well plates and incubated for 1h at 37°C. The monolayers were then overlaid with 100 µl DMEM containing 1% methyl cellulose. After incubation for 24 h at 37°C the overlay was removed and the monolayer was fixed and stained with 0.5% cristal violet. The highest dilution of the serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was first pretreated with an equal volume of 0.1M 2-ME in saline (Scott and Gershon, 1970).

**Assessment of foot pad swelling**
Mice were inoculated with 30 pfu of LCMV-WE into both hind foot pads and foot pad swelling was assessed daily with a spring-loaded caliper (Castelmur et al., 1993). In vivo anti-CD4 treatment was performed at day 6 and 8 after inoculation using the monoclonal antibody YTS 191.1 (Cobbold et al., 1984).

**Protection of mice from replication of recombinant vaccinia virus**
Mice were immunized with 2x10^6 pfu VSV and challenged i.p. 8 days later with 5x10^6 pfu recombinant vaccinia virus expressing VSV-glycoprotein (VaccGIND). Vaccinia titers in ovaries were determined 5 days later as described previously (Binder and Kündig, 1991). Titers are shown as log10 pfu per animal.
Results

*Induction of virus-specific Th cells*

In some experimental systems B-cells have been shown to be crucial for efficient T helper cell induction (Janeway et al., 1987; Kurt-Jones et al., 1988; Lawton et al., 1972; Liu et al., 1995; Ron et al., 1981; Ron and Sprent, 1987; van Ewijk et al., 1988). In addition, CD40 is not solely expressed on B-cells but also on dendritic cells and follicular dendritic cells (Banchereau et al., 1994). We addressed therefore the question whether CD40-CD40L interaction or the presence of B cells serving as antigen presenting cells is required for the induction of virus-specific Th cells in vivo. We compared the induction of LCMV-specific Th cells in vivo in CD40-, CD40L-deficient, normal C57BL/6 and IgM-deficient mice. Since it can be expected that B-cells do not serve efficiently as APCs in the absence of proper activation and proliferation induced by the CD40-CD40L interaction, IgM-deficient mice that do not have peripheral B-cells (Kitamura et al., 1992) were included in this comparison. This made possible the distinction between Th cell induction without the CD40-CD40L interaction and Th cell induction without B-cells serving as antigen presenting cells.

Induction of LCMV- or P13-specific Th cells in vivo was comparable in CD40L-, CD40- and B cell-deficient mice and in normal C57BL/6 mice as defined by specific proliferation in response to both antigens (Fig.1). These results demonstrate that LCMV-specific Th cells are induced in vivo independent of a functional CD40-CD40L interaction. Since induction of LCMV-specific Th cells in CD40-deficient as well as in CD40L-deficient mice and B-cell-deficient mice was similar, Th cells can apparently be stimulated in vivo without crucial involvement of B-cells or of a functional CD40-CD40L-interaction after LCMV infection.
Fig. 1 LCMV-specific proliferation of purified Th cells. CD40L-deficient, CD40-deficient, IgM-deficient and C57BL/6 mice were immunized with LCMV and 14 days later LCMV-specific proliferation of purified CD4+ T cells was determined using either UV-inactivated LCMV (WE) or peptide 13 (P13) as LCMV-specific antigens (concentrations of stimulating antigens are described in Materials and methods). P13 is a LCMV-GP-derived peptide recognized by CD4+ T cells. Stimulation indices were calculated in relation to proliferation in medium control. Background cpm counts in medium control were always around 2000 cpm. Two of four equivalent experiments are shown.
In addition, cytokine secretion patterns were analyzed in the supernatants recovered from the proliferation assays performed with LCMV-primed CD4+ T cells from CD40-, CD40L-deficient or control C57BL/6 mice (Fig.2). IL-2 and IFNγ were secreted in comparable amounts by CD4+ T cells originating from CD40-, CD40L-deficient or control mice whereas no IL-4 could be detected in the same supernatants (Fig.2). Thus, the Th1 cytokine secretion pattern which is normally observed for LCMV-specific Th cells is not influenced by the absence of functional CD40-CD40L interactions.

Fig.2 LCMV-specific cytokine secretion. In vivo LCMV-primed CD40-, CD40L-deficient or control CD4+ T cells were restimulated in vitro with the LCMV-glycoprotein-derived I-A\(^b\)-binding peptide P13 (circles) or without antigen (medium control (squares)). IL-2 content in the supernatants (5000 EM units correspond to 30 U IL-2/ml) was analyzed 24 hrs after restimulation (upper panel) whereas IFNγ (OD=0.5 corresponds to 500 U IFNγ/ml) and IL-4 levels (OD=0.5 corresponds to 25 U IL-4/ml) were assessed 60 hrs after restimulation (middle and lower panels). Recombinant IL-4 (open circles) served as positive control in the IL-4 ELISA. Each line represents a separate proliferation assay from an individual mouse. One of three equivalent experiments is shown.
In a second experimental approach, induction of LCMV-specific Th cells was assessed in CD40-deficient mice and in heterozygous littermates (both being competent for CD40L on Th cells) by measuring T helper cell function upon transfer of primed Th cells into CD40-competent naive recipients. Thus, CD40L competent cells were primed in a CD40-deficient environment. Adoptive transfer of CD40L-positive, LCMV-primed Th cells into naive recipients with normal CD40+ B cells restores functional T-B cooperation. The conditions for challenging the recipient mice were chosen such that only Th cells already primed in the CD40-deficient donors were able to induce a significant isotype switch in the recipient. To assess the importance of B cells for the induction of Th cells, B cell-deficient mice were included in the experiment. CD40-deficient, B cell-deficient and control mice were infected with LCMV and eighteen days after infection spleen cells were transferred into naive recipients. This was followed by a challenge immunization with LCMV-DNP. DNP-specific IgG antibodies were only significantly induced after challenge if LCMV-specific Th cells had been induced in the donor animals prior to transfer (Fig.3). Since B cells in the recipients express CD40, equivalent DNP-specific IgG titers in recipients that received primed Th cells from immunized CD40-deficient mice or from heterozygous littermates revealed that induction of LCMV-specific Th cells was not dependent on a functional CD40-CD40L interaction in vivo (Fig.3 A, B). Similarly, equivalent IgG titers in recipients that received Th cells from LCMV-immunized B cell-deficient mice or B cell-competent C57BL/6 mice indicated that induction of LCMV-specific Th cells was not dependent on the presence of B cells serving as antigen presenting cells (Fig.3 C+D).
Fig. 3. Cognate help to CD40-expressing B-cells after adoptive transfer of LCMV-primed Th cells. CD40-deficient mice (triangles) and heterozygous littermates (circles) as well as IgM-deficient mice (diamonds) and control mice (circles) were immunized with LCMV. 20 days after infection spleen cells were adoptively transferred into CD40 competent naive recipients and the recipients as well as naive CD40 competent control animals (squares) were challenged with LCMV-DNP. After 9 days, DNP-specific IgG titers were determined by ELISA from 30-fold prediluted sera. Each line represents one individual mouse. One of three similar experiments is shown.
To confirm that the transferred CD4+ T cells were alone responsible for the enhanced DNP-specific IgG titers, purified CD4+ T cells from LCMV-primed CD40-deficient mice or heterozygous littersmates were transferred into naive C57BL/6 recipients which were challenged by injection of LCMV-DNP. DNP-specific IgG titers were determined 10 days later (Fig. 4 A, B). In addition, transfer of unseparated spleen cells from LCMV-primed CD40-deficient donors into naive C57BL/6 recipients served as positive control (Fig. 4 C), whereas unmanipulated, unprimed C57BL/6 mice served as negative control. Both CD4+ T cells originating from either LCMV-primed CD40-deficient mice or heterozygous littersmates were able to comparably provide help for CD40-competent B cells (Fig. 4 A, B), albeit whole spleen cell transfers resulted in a more pronounced enhancement of DNP-specific IgG titers as compared to purified CD4+ T cells.

Thus, three independent assays showed that the induction of LCMV-specific Th cell responses was not limited by CD40-CD40L interactions and was independent of B cells acting as critical APCs.

Fig. 4 Cognate help to CD40-expressing B cells after adoptive transfer of purified, LCMV-primed CD4+ T cells. CD40-deficient mice (triangles) and heterozygous littersmates (circles) were immunized with 200 pfu LCMV i.v.. 14 days later CD4+ T cells were purified and adoptively transferred into CD40-competent, naive recipients (A, B). In addition, spleen cells from LCMV-primed CD40-deficient donors (diamonds) were transferred into CD40-competent naive recipients (C). All recipients as well as naive control animals (squares) were challenged with LCMV-DNP and 10 days later DNP-specific IgG titers were determined in 90-fold prediluted sera. Each line represents an individual recipient. One of two similar experiments is shown.
**Th cell effector function: cognate help to B-cells**

It has already been demonstrated in detail that T-dependent IgG humoral immune responses are strictly dependent on a functional CD40-CD40L interaction and that a missing ligand in this complex totally abrogates germinal center formation as well as immunoglobulin isotype switch and generation of memory B-cells (Banchereau et al., 1994; Foy et al., 1994; Foy et al., 1993; Gray et al., 1994; Lane et al., 1993; Lederman et al., 1992a; Lederman et al., 1992b; Noelle et al., 1992; Parker, 1993; Van den Eertwegh et al., 1993). To ensure that Ig responses to LCMV as a model viral antigen require CD40-CD40L engagement, we infected CD40L-deficient or C57BL/6 mice with LCMV and then measured antiviral primary antibody responses. VSV was used as a second viral model antigen.

Infection of normal mice with LCMV leads to a pronounced IgG response specific for the nucleoprotein of LCMV (LCMV-NP) (Fig.5). In contrast, no LCMV-NP-specific IgG titers were obtained using CD40-deficient mice (Fig.5). Thus, CD40-CD40L interaction is crucial for isotype class switching in response to LCMV-infection.
Fig. 5 LCMV-NP specific IgG antibody response. CD40L-deficient (circles), CD40-deficient (triangles) and normal C56BL/6 (squares) mice were immunized with LCMV. LCMV-NP specific IgG titers were determined 7, 10 and 21 days after infection for CD40L-deficient and normal C56BL/6 mice. LCMV-NP specific IgG titers in CD40-deficient and normal C57BL/6 mice were assessed 20 days after LCMV infection in 30-fold prediluted sera. Each line represents one individual mouse. Variations were smaller than 2 dilution steps. One of three comparable experiments is shown.
In addition, we analyzed virus neutralizing antibody responses in CD40L-deficient and normal mice after infection with VSV. VSV infection of mice normally induces a type I T-independent neutralizing IgM-response followed by a T-dependent neutralizing IgG response (Bachmann et al., 1995; Bachmann et al., 1993; Leist et al., 1987) As expected control C57BL/6 mice mounted high neutralizing IgM and IgG responses (Fig.6). In contrast, CD40L-deficient mice mounted a neutralizing T-independent IgM response but no VSV-specific IgG was detected upon VSV infection (Fig.6). The same results were obtained using CD40-deficient mice (data not shown). These data corroborate previous findings in nonviral systems, demonstrating that cognate T-B cooperation leading to Ig class switching is critically dependent on functional CD40-CD40L interactions.

Fig.6 VSV neutralizing antibody response. CD40L-deficient and C57BL/6 mice were immunized with VSV. VSV-neutralizing IgM titers (squares) and VSV-neutralizing IgG titers (triangles) were determined from 40-fold prediluted sera 4, 6, 8, 12 and 20 days after infection. Each line represents one individual mouse. Variations were smaller than 2 dilution steps. One of three comparable experiments is shown.
**Th cell effector function: inflammatory reaction**

Infection of normal mice with LCMV in the hind footpads induces a local inflammatory reaction consisting of two sequential swelling phases. A pronounced swelling reaction mediated by CD8+ T cells is observed 7-10 days after infection; this is followed by a CD4+ T cell mediated swelling phase declining around day 14-16 after infection (Leist et al., 1987; Moskophidis and Lehmann, 1989). The second phase of the swelling reaction exhibits no sharp peak but has the shape of a shoulder. We analyzed whether or not the second swelling phase mediated by LCMV-specific CD4+ T cells was dependent on CD40-CD40L interactions. CD40-deficient mice as well as heterozygous littermates were infected with LCMV in both hind footpads and the subsequent swelling reaction was monitored daily (Fig. 7A). In fact, there was a tendency noted that CD40-deficient mice exhibited a slightly increased maximal early CD8-dependent peak response and that subsequently the CD4-dependent shoulder tended to be slightly higher. Thus, neither the CD8+ T cell mediated swelling phase nor in the CD4+ T cell mediated swelling phase was dependent upon a functional CD40 molecule. Compatible results were obtained with CD40-deficient mice (Fig. 7B). This indicates comparable abilities to mount LCMV-specific inflammatory reactions in the presence or absence of CD40-CD40L interaction. In order to confirm that the second phase of the swelling reaction is actually CD4+ T cell mediated, C57BL/6 mice were treated with a CD4+ T cell-depleting monoclonal antibody at day 6 and 8 after inoculation with LCMV. Foot pad swelling was less marked and the subsequent decline was much more rapid compared to untreated controls (Fig. 7B).
Fig. 7 LCMV-specific foot pad swelling reaction. In (A) CD40-deficient mice (triangles) and heterozygous littermates (squares) were immunized into the footpads with 200 pfu of LCMV and the local LCMV-specific foot pad swelling reaction was daily monitored. In (B) LCMV-specific foot pad swelling reaction was similarly analyzed in CD40L-deficient (circles) and normal C57BL/6 mice (squares) as well as in anti-CD4 treated C57BL/6 mice (diamonds). Each line represents one individual mouse.
Th cell effector function: antiviral protection

A third Th cell effector function was analyzed in CD40L-deficient and normal mice. VSV primed C57BL/6 mice have been shown to rapidly control replication of a recombinant vaccinia virus expressing the glycoprotein of VSV (VaccG\textsubscript{IND}). This antiviral protective mechanism is virtually exclusively mediated by IFN\(\gamma\) and TNF\(\alpha\) which are secreted by VSV glycoprotein-specific CD4\(^+\) T cells (Binder and Kündig, 1991; Kündig et al., 1993a). Neither CD8\(^+\) T cells nor VSV-neutralizing antibodies have been shown to be protective in this infection. Because G\textsubscript{IND} is not expressed in the VaccG\textsubscript{IND} envelope, VSV-specific antibodies cannot bind or neutralize VaccG\textsubscript{IND} recombinant virus. CD40L-deficient and normal mice were infected with VSV and 8 days later the VSV primed mice as well as naive control animals were challenged intraperitoneally by infection with VaccG\textsubscript{IND}. Vaccinia titers in the ovaries were determined 5 days after challenge (Fig.8). As with the foot pad swelling results (Fig.7A), we did not observe a reduced CD4\(^+\) T cell mediated antiviral effector function in CD40L-deficient as compared to CD40L-competent mice.

![Graph showing vaccine titers in ovaries](image)

Fig.8 CD4\(^+\) T cell dependent protection against challenge infection with recombinant vaccinia virus. CD40L-deficient and normal C57/BL6 mice were immunized with 2x10\(^6\) pfu of VSV and 8 days later these primed mice as well as naive control mice were challenged i.p. with 5x10\(^6\) pfu VaccG\textsubscript{IND}. Vaccinia titers in ovaries were determined 5 days after challenge infection. One of two experiment is shown.
Discussion

Many reports have focussed on the key importance of the CD40-CD40L interaction for the generation of humoral immune responses (Banchereau et al., 1994; Foy et al., 1994; Lederman et al., 1992a; Lederman et al., 1992b; Noelle et al., 1992; Parker, 1993). This was confirmed here for antiviral IgG immune responses. In contrast to most previous reports, this study examined in addition the role of CD40-CD40L interaction on Th cell activation, proliferation and effector functions. Surprisingly, neither Th cell induction nor Th cell effector functions other than cognate help for B cells were compromised by the lack of the CD40- or the CD40L molecules.

CD40 is expressed on different APCs such as dendritic cells and B-cells and macrophages (Clark et al., 1992; Freudenthal and Steinmann, 1990; Galy and Spits, 1992; Ling et al., 1987; Schriever et al., 1989). In addition, it has been shown that CD40-CD40L interaction induces upregulation of MHC class II, B7 and ICAM-1 on dendritic cell lines which potentiate the stimulatory capacity of the APC (Sallusto and Lanzavecchia, 1994). The CD40-CD40L interaction-dependent upregulation of these and maybe other accessory molecules on the surface of the APC signals then back to the T cell, a process which may be supported by interleukins in addition. Thus, it could be expected that activation and proliferation of specific Th cells would be impaired in the absence of the CD40-CD40L interaction as in fact has been recently reported (Grewal et al., 1995; van Essen et al., 1995). However, we report here that antigen-specific Th cells are induced normally in vivo in the absence of a functional CD40-CD40L interaction after two different virus infections; hence the formation of this receptor-ligand pair is apparently not critical for the activation of naive virus-specific Th cells. Since B-cell activation and proliferation seems to depend critically upon the presence of cognate T-help (Foy et al., 1994; Foy et al., 1993; Han et al., 1995; Nishioka and Lipsky, 1994), absence of CD40-CD40L interaction will interfere with proliferation of specific B-cells and therefore reduce class II associated antigen presentaion by specific B cells. Because it has been reported that antigen presentation by B-cells is important for the normal induction of T-helper cells in several systems (Janeway et al., 1987; Kurt-Jones et al., 1988; Lawton et al., 1972; Liu et al., 1995; Ron et al., 1981; Ron and Sprent, 1987; van Ewijk et al., 1988), our results suggest that either B-cell proliferation and activation is impaired to a smaller degree than expected in the absence of CD40-CD40L interaction, or, alternatively and more likely, that the presence of specific B-cells plays only a minor if any role for the induction of T-helper cells with the infectious virus-derived antigens studied here (Cerny et al., 1986). This latter possibility is supported by the fact, that Th cells primed in the absence of B cells proliferated normally and could mediate isotype switch after adoptive transfer (Fig.3 C+D). These findings are in contrast to recently presented data (Grewal et al., 1995; van Essen et al., 1995). Grewal et al. (Grewal et
al., 1995) showed a significant impairment of KLH- or HEL-specific T cell priming in mice lacking CD40 ligand. This impairment was evidenced by strongly reduced recall proliferation responses and by a failure of adoptively transferred CD40L-deficient T cells to expand in vivo after antigenic challenge. In contrast to these findings, van Essen et al. observed normal KLH-specific Th cell induction in CD40-deficient mice, but described these KLH-primed T cells as being qualitatively different from T cells primed in a CD40-competent environment; they were apparently not able to provide help to B cells. These differences concerning the importance of a functional CD40-CD40L interaction for the induction and effector functions of T cells between the virus models used in this study and the soluble KLH antigen system used by van Essen et al. and by Grewal et al. probably reflect different critical in vivo mechanisms governing both the induction and effector functions of T cells. There are at least two possible explanations that might account for these differences: 1. The efficient activation of T cells following a viral infection such as with LCMV might over-ride some of the more subtle requirements for responses to soluble protein antigens such as KLH. In addition, the failure of CD40-deficient mice to resolve a Leishmania infection (H. Kikutani, personal communication), despite exhibiting a Leishmania-resistant genetic background, could be due to the lack of CD40 signals to macrophages or dendritic cells which then could lead to a deficit in IL-12 production and therefore to an impaired Th1 development. Additional evidence for a requirement of a functional CD40-CD40L interaction for IL-12 induction in vivo and hence the induction of a Th1 response has recently been presented by Stüber et al. (Stüber et al., 1996). In a viral infection the direct secretion of IFNγ and possibly other interleukins by T cells may render this pathway less critical. 2. Alternatively, B cells may be of key importance as APCs for T cell induction in a soluble antigen system in contrast to virus model infections. There still exists an unresolved controversy on the issue whether B cells play a central role in the initiation of T cell immune responses. Even studies using the same experimental antigen such as KLH as soluble antigen and B cell-deficient mice revealed different results: Epstein et al. (Epstein et al., 1995) reported successful T cell priming in B cell-deficient mice using KLH as antigen whereas Liu et al. (Liu et al., 1995) failed to obtain T cell priming in B cell-deficient mice using either KLH or OVA as soluble protein antigens. Constant et al. (Constant et al., 1995c) demonstrated that mice lacking B cells were impaired in their priming of T cells to protein but not to peptide antigens. This dominant role of B cells as APCs found in several experimental systems using soluble antigens is possibly abrogated if the CD40 signaling pathway is not functional. We deliberately chose an experimental system where T cell induction is not dependent on B cells functioning as APCs and analyzed the role of
CD40-CD40L interaction in T cell priming and Th cell effector functions independently of the presence or absence of B cells.

The effector functions of Th cells are differentially influenced by CD40-CD40L interaction. Thymus-dependent Ig-isotype switched humoral immune responses are completely abrogated in CD40- or CD40L-deficient mice whereas thymus-independent IgM-responses to VSV are comparable to normal mice; the latter apparently do not require a functional interaction between CD40 and its ligand. Although T-helper cells do not induce antibody isotype switching in B-cells in the absence of CD40-CD40L interaction, CD4+ T cells could be functionally primed in CD40-deficient mice since upon transfer they were able to provide help for CD40-competent B cells to undergo isotype switch. These findings are in contrast to the recent data of van Essen et al. (van Essen et al., 1995) showing that KLH-specific T cells primed in a CD40-deficient environment are not able to help CD40-competent B cells. Although the experimental setups are not identical, it can be concluded that Th cell priming by virus infection seems to be considerably more efficient in a CD40-deficient environment than is possible for soluble protein antigens.

Not only were induction, proliferation and cytokine production of specific T-helper cells normal in the absence of CD40-CD40L interaction but also effector functions of T-helper cells other than mediating Ig isotype switching were largely normal in the absence of CD40 or CD40L. CD4+ T cell mediated inflammatory reaction upon LCMV infection was not impaired in CD40- or CD40L-deficient mice.

In addition, Th cell-dependent antiviral protection was not impaired by the lack of CD40-CD40L interaction: activated VSV-specific Th cells in CD40L-deficient mice were able to inhibit the replication of VSV-G recombinant vaccinia virus by a cytokine mediated effector function; this protective Th cell function has been shown to be mediated by release of IFNγ and TNFα by activated CD4+ Th cells (Binder and Kündig, 1991). It has recently been suggested, that CD40L may exert a direct protective effect against vaccinia virus (Ruby et al., 1995). Our data show that Th cells can efficiently protect against vaccinia virus replication also in the absence of CD40L, suggesting that this parameter is not limiting under the conditions tested in vivo.

In conclusion, these results reveal a dichotomy in the role of the CD40-CD40L interaction in Th cell effector functions: the functional interaction is of critical importance in T-B cell cooperation for the induction of thymus-dependent humoral immune responses but the same interaction is not limiting for Th cell mediated effector functions such as virus-induced inflammatory reactions or antiviral protection, which probably depend on interactions between Th cells with macrophages and/or antigen presenting cells. Our data strongly suggest that CD40-CD40L interaction divides anti-viral Th cell effector functions in two categories: One
category consisting of cognate T help mediating B-cell activation followed by Ig class switching which is strictly dependent on a functional CD40-CD40L interaction; and a second category, represented in this report by virus-induced, CD4+ T cell-mediated inflammatory reaction as well as antiviral protection is largely independent of a functional CD40-CD40L interaction. Similarly, activation and proliferation of Th cells, albeit not a Th cell effector function involved in T-B cooperation, may be placed in this second category. Thus, CD40-CD40L interaction is critically and probably unidirectionally-needed for B-cell activation followed by Ig class switch, but in addition is possibly of importance for macrophage/dendritic cell activation especially in the case of nonviral antigens.
Chapter 4:

**Virus-specific MHC Class II-restricted TCR transgenic mice: Implications of elevated Th cell frequencies on humoral and cellular immune responses after viral infection**

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Abbreviations used in this paper: BSA, bovine serum albumin; DNP, dinitrophenol; P13, peptide 13; P61, peptide 61; ER, endoplasmic reticulum; GP glycoprotein; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; TCR, T cell receptor; pfu, plaque forming units; VSV, vesicular stomatitis virus.
Abstract

A transgenic mouse expressing a MHC class II-restricted T cell receptor with specificity for a lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived helper cell epitope was generated to characterize the role of LCMV-specific CD4+ T cells upon infection. Analysis of LCMV-specific humoral immune responses revealed that the TCR transgenic CD4+ T cells efficiently mediated help for the production of LCMV-glycoprotein-specific isotype-switched antibodies as well as for the production of IgG antibodies specific for a non-LCMV-related B cell epitope which was covalently coupled to the appropriate LCMV-glycoprotein Th cell epitope. In contrast, LCMV-glycoprotein-specific TCR transgenic mice exhibited a drastically reduced ability to provide help for the generation of LCMV-nucleoprotein-specific antibody responses as compared to normal control mice, indicating that intramolecular/intrastructural help is restricted to viral surface B cell antigens. TCR transgenic mice exhibiting a profound skewing towards the CD4+ T cell compartment showed a delayed and in efficiency reduced LCMV-specific CD8+ cytotoxic T cell response leading to an impaired and delayed LCMV clearance after infection. This demonstrates that functional LCMV-specific CD4+ T cells - even if present at extremely high frequency - can not directly mediate protective immunity against LCMV. As opposed to infection with non-cytopathic LCMV, TCR transgenic mice were protected against infection with cytopathic recombinant vaccinia virus expressing the LCMV glycoprotein. These results further strengthen the different requirements in terms of immune effector functions which are involved in protective immunity to different viral infections. In addition, the ability to physically and functionally observe LCMV-specific CD4+ T cells in vivo offers a unique opportunity of characterizing virus-specific CD4+ T cells responses in an infectious disease model.
Introduction

Infection of mice with noncytopathic lymphocytic choriomeningitis virus (LCMV) induces a vigorous cellular and humoral immune response (Buchmeier et al., 1980; Moskophidis and Lehmann-Grube, 1983; Moskophidis and Lehmann-Grube, 1984; Zinkernagel, 1993). The cellular mechanisms responsible for the control of LCMV infection have been studied in great detail and have revealed that perforin-mediated cytotoxicity by LCMV-specific CD8+ effector T cells is important for the resolution of primary infections (Kägi et al., 1994a). Studies in transgenic mice expressing a MHC class I-restricted TCR specific for the LCMV glycoprotein derived peptide gp33-41 demonstrated that the transgenic mice were able to resolve low dose LCMV infection with drastically enhanced kinetics whereas infection with high doses of LCMV resulted in rapid generation of CTL escape mutants due to the high mutation rate of the RNA virus LCMV (Pircher et al., 1990a). Infection of normal mice with high doses of the widely replicating LCMV-Docile isolate was shown to induce exhaustion of LCMV-specific CTLs ultimatively leading to a life-long virus carrier status (Moskophidis et al., 1993).

A transient general immunosuppression is observed in normal mice 8-10 days after low dose LCMV infection due to the fact that LCMV-infected lymphoid cells in secondary lymphoid organs are efficiently lysed by CD8+ effector T cells thus leading to destruction of the follicular structures and of the architectural integrity of secondary lymphoid organs which are indispensable for the induction of immune responses (Leist et al., 1988; Odermatt et al., 1991).

The induction of in vivo protective LCMV-specific CTL responses during acute LCMV infection has been shown to occur independently of CD4+ T cells although a decrease in efficiency of CTL induction by a factor of 3 to 9 was documented in the absence of CD4+ T cells (Leist et al., 1989b). In contrast, the presence of CD4+ T cells (and B cells) is crucially involved in long term protective immunity against LCMV infection and MHC class II-deficient mice or B cell-deficient mice show spontaneous reappearance of viral titers 2 to 3 months upon infection after they had apparently cleared the virus during the acute phase of infection (Thomsen et al., 1996). In addition, viral clearance after infection with certain LCMV isolates which cause prolonged infection requires long-term CTL responsiveness and may take 2-3 months after initial infection. In this case, CD4+ T cells were able and necessary to sustain LCMV-specific CTL responsiveness over this long time period. Absence of CD4+ T cells resulted in exhaustion of LCMV-specific CTLs and thus in the induction of a virus carrier status (Matloubian et al., 1994). In line with this, it has been shown that CD4-deficient mice are susceptible to CTL exhaustion at lower doses of the initial virus inoculum than CD4-competent mice (Battegay et al., 1994). In addition, it has been reported that the absence of CD4+ T cells during acute LCMV
infection negatively influences CTL memory in terms of LCMV-specific CTL precursor frequencies and thus renders these mice more susceptible to secondary LCMV(-related) infections (von Herrath et al., 1996).

Humoral immune responses against LCMV are T-dependent and therefore require help by CD4+ T cells for both the induction of IgM responses and isotype-switched IgG responses (Ahmed et al., 1988; Leist et al., 1987). LCMV-specific humoral immune responses may be divided into two subclasses: into LCMV-binding antibody responses and into LCMV-neutralizing antibody responses. Whereas LCMV NP- or GP-binding isotype switched antibody responses are readily induced at high titers upon infection and are measurable already 8 days after infection, LCMV-neutralizing antibody responses usually appear only very late after infection (>60 days after infection) and show only moderate titers. Interestingly, neutralizing antibody responses are induced with enhanced kinetics in the absence of CD8+ T cells (Battegay et al., 1993).

In order to investigate LCMV-specific CD4+ T cells responses in vivo on a defined cellular level with respect to antiviral cellular and humoral immune functions, a transgenic mouse was generated expressing a MHC class II-restricted TCR with specificity for an immunodominant LCMV GP-derived helper cell epitope P13 (GP 61-80) (Oxenius et al., 1995). Here we report on the generation of the TCR transgenic mice and on their phenotypical and functional in vivo characterization.
Material and Methods

1. Mice
Inbred C57BL/6 (H-2\textsuperscript{b}) mice were obtained from the breeding colony of the Institut für Zuchthygiene, Tierspital Zürich, Switzerland. The generation of CD8-deficient mice was described before (Fung-Leung et al., 1991b). The generation of MHC class II restricted TCR transgenic mice is described below. Mice were bred in SPF facilities.

2. Viruses and recombinant proteins
The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube, Hamburg, Germany and grown on L929 cells (ATCC CRL 1) with a low multiplicity of infection.
Recombinant vaccinia viruses expressing LCMV GP or LCMV NP have been described (Schulz et al., 1989; Whitton et al., 1988). Recombinant viruses were grown at low multiplicity of infection on BSC cells and plaqued on BSC cells.
The recombinant baculovirus expressing the LCMV nucleoprotein has been previously described (Matsuura et al., 1987) The recombinant baculovirus was derived from nuclear polyhedrosis virus and was grown at 28°C in Spodoptera frugiperda cells in spinner cultures in TC-100 medium. Recombinant proteins were produced as previously described (Bachmann et al., 1994a).
The recombinant bacteria expressing the LCMV GP2 protein have been previously described (Weidt et al., 1994). IPTG-induced expression of the recombinant protein in E.coli cells was followed by purification via affinity chromatography as previously described (Weidt et al., 1994). Protein concentrations were determined by UV spectroscopy.

3. Peptides
Peptides were purchased from NMR Reutlingen, Germany or from Neosystem, Strasbourg, France. The following peptides were used:
P13 (GLNGPDIYKGYQFKSVEFD) (LCMV GP, I-A\textsuperscript{b})
P61 (SGEGWPYIACRTSVVGRAWE) (LCMV NP, I-A\textsuperscript{b})
GP33 (KAVYNFATM) (LCMV GP, D\textsuperscript{b})

4. Generation of TCR transgenic mice
a) Generation of a LCMV GP-specific CD4\textsuperscript{+} T cell clone
C57BL/6 mice were infected intravenously with 200 pfu LCMV WE and 11 days later CD4\textsuperscript{+} T cells were purified from single cell spleen suspensions by MACS sorting according to the protocol of the supplier (Miltenyi Biotec, Germany). CD4\textsuperscript{+} T
cells were restimulated in vitro for several rounds in 14 day intervals with irradiated autologous spleen cells and the LCMV GP-derived peptide P13 (Oxenius et al., 1995), finally leading to a LCMV-GP-specific CD4⁺ T cell line. Limiting dilution of CD4⁺ T cells yielded several LCMV-specific CD4⁺ T cell clones one of which (clone 1) was chosen for further characterization. Clone 1 was specific for the LCMV GP-derived peptide P13 (amino acids 61-80) and restricted to the I-Aβ MHC class II molecule. The TCR variable regions of clone 1 were encoded by the Vα2 and Vβ8.3 elements as identified by FACS analysis using the Vα2-specific monoclonal antibody B20.1 (Pircher et al., 1992) and the Vβ8.3-specific monoclonal antibody 7G8 (Foerster et al., 1995). Sequencing of the corresponding variable region genes revealed that the Vα variable region gene was composed a Vα2.3-JαDK1 rearrangement and that the Vβ variable region gene was composed of a Vβ8.3-Jβ2.5 rearrangement (EMBL nucleotide sequence database, accession numbers Y11102 and Y11103).

b) Generation of the TCR transgenic mouse

The variable region genes of clone 1 were amplified by PCR using genomic DNA as a template and the following primer combinations.

For Vα2: 5'-TGACCCGGGAGCTTCAGTCTAGGAGGAATG-3'
and 5'-TATGCGGCCGCTATCAGGTACTTACTGGGG-3'
For Vβ8.3 5'-GGCCTCGAGCATACAAGAGCCTGACTTGGTCGAGATG-3'
and 5'-TGCCCGCGGCTGAGAACGCGCACGTGGGG-3'

The primers used for Vα2 variable gene amplification deliberately introduced a 5' flanking Xmal site (20 bp 5' of the ATG start codon) and a 3' flanking NotI site (30 bp 3' of the splice donor sequence of JαDK1). The primers used for Vβ8.3 variable gene amplification similarly introduced a 5' flanking Xhol site and a 3' flanking SacII site. After sequence analysis to ensure fidelity during PCR amplification, the Xmal-NotI Vα2 fragment and the XhoI-SacII Vβ8.3 fragment were cloned into previously described genomic TCR expression cassette vectors (Katz et al., 1993). The resulting Vα2-PαTRIS was digested with SalI to liberate the 17.5 kbp Vα2 injection fragment whereas the 19 kbp Vβ8.3 injection fragment was excised from the resulting Vβ8.3-PβTRIS construct by digestion with Kpnl.

TCRαβ transgenic mice were generated at BRL (Biological Research Laboratories, Füllinsdorf, Switzerland) by coinjection of the Vα2 and Vβ8.3 fragments into fertilized C57BL/6 eggs. Threefold backcrossing of a founder mouse exhibiting multiple integrations on different chromosomes to C57BL/6 mice yielded two TCRαβ transgenic mouse lines (SmarTa1 and Smarta2) which were used for experiments.
5. Cytofluorometric analyses
The following monoclonal antibodies were used for analysis of thymocytes and peripheral lymphocytes: biotinylated 7G8, specific for VP8.3 (a gift from Dr. I. Förster, (Foerster et al., 1995)); phycoerythrin-conjugated B20.1, specific for Vα2 (purchased from Pharmingen); fluorescein-conjugated anti-CD8 (purchased from Gibco, BRL) and fluorescein-or phycoerythrin-conjugated anti-CD4 (purchased from Gibco, BRL). Tricolour-conjugated streptavidin or anti-CD4 were purchased from Caltag Laboratories.

Thymocytes were stained with phycoerythrin-labeled anti-CD4, FITC-labeled anti-CD8 and biotinylated 7G8 (or with phycoerythrin-labeled B20.1, FITC-labeled anti-CD8 and tricolour-labeled anti CD4) for 30 min. on ice, washed once in PBS containing 2% FCS and 0.1% NaN3. Then, thymocytes stained with anti-CD4, anti-CD8 and 7G8 were incubated with tricolour-conjugated streptavidin for 30 min on ice and washed once more before analysis.

Lymph node cells were stained either with phycoerythrin-labeled anti-CD4 and fluorescein-labeled anti-CD8 or with fluorescein labeled anti-CD4, phycoerythrin-labeled anti-Vα2 and biotinylated 7G8 for 30 min. on ice. After washing in PBS containing 2% FCS and 0.1% NaN3, triple stained cells were incubated for 30 min. on ice with tricolour-conjugated streptavidin and washed once more before analysis.

Identical procedures were applied for the staining of PBLs but the washing buffer was supplemented with 20 mM EDTA and before the last washing step, red blood cells were subjected to lysis (lysis solution, Becton-Dickinson).

Flow cytometry was performed on an FACSstar Plus flow cytometer (Becton-Dickinson).

6. Proliferation
6x10⁴ spleen cells from TCR transgenic mice or from negative littermates were incubated in flat bottom 96-well plates with 3-fold serial dilutions of LCMV (MOI=2 at highest concentration), LCMV GP-derived peptide P13 (1μg/ml at the highest concentration) or LCMV NP-derived peptide P61 (1μg/ml at the highest concentration) in the presence of 6x10⁵ irradiated (2000cGy) C57BL/6 spleen cells for 3 days. Proliferation was assessed by incorporation of ³H-thymidine (25μCi/well).

7. Cytokine analysis
Supernatants of proliferation assays as described above were analyzed for IL-2 content (24 hrs after restimulation), IFNγ content (60 hrs after restimulation) and IL-4 content (60 hrs after restimulation). IL-2 was determined using the IL-2-dependent cell line CTLL-2. Quantification of viable cells was performed by AlamarBlue™
colour reaction (Biosource, International) and measured by fluorescence emission at 590 nm using the CytoFluor™ 2350 (Millipore) fluorimeter. IFNγ and IL-4 were assessed by ELISA as described (Chirmule, 1991; Pruslin, 1991)

8. Adoptive transfer of naive TCR transgenic T cells

10^6 splenocytes from TCRαβ transgenic mice were adoptively transferred intravenously into normal negative littermates which were infected intravenously with 200 pfu LCMV WE either 2 days before transfer, at the timepoint of transfer or 3 days after transfer. In vivo clonal expansion of transferred TCR transgenic CD4+ T cells was analyzed 5, 7, 8, 11, 15 and 29 days after adoptive transfer by triple staining of peripheral blood lymphocytes with anti-CD4, anti-Vα2 and anti-Vβ8.3 monoclonal antibodies and subsequent FACS analysis.

9. ELISA

The LCMV nucleoprotein-specific enzyme-linked immunosorbent assay (ELISA) has been described previously (Battegay et al., 1993). 96-well plates (Petra Plastik, Chur, Switzerland) were incubated with LCMV nucleoprotein (0.01 µg/well) in 0.1M NaH₂PO₄, pH 9.4 at 4°C. Plates were then preincubated with 2% bovine serum albumine in phosphate-buffered saline for 2 h and washed, and serial dilutions of serum samples (30-fold prediluted) were added to the wells and incubated for 90 min. Plates were washed and incubated with horse raddish peroxidase-labeled goat anti-mouse IgG (Sigma). After 90 min, plates were washed and developed with ABTS (5 mg of 2,2'-azino-di-3-ethyl-benzthiazolinsulfonate and 20 µl of H₂O₂ in 50 ml of NaHCO₃ [pH4]). Optical densities were determined at 405 nm.

ELISA-measurement of LCMV-GP-specific or DNP-specific IgG titers was performed similarly. ELISA-plates were either coated with 0.1 µg/well purified recombinant LCMV-GP2 or with 0.1 µg/well of ovalbumin covalently coupled to DNP (Charan et al., 1986; Mishell and Shiigi, 1980).

In vivo depletion of CD4+ and CD8+ T cells was achieved by administration of the respective depleting monoclonal antibodies YTS 169.4 or YTS 191.1 (Cobbold et al., 1984).

10. Cytotoxic T cell response

Ex vivo cytolytic activity of spleen cells was determined as previously described (Zinkernagel et al., 1985). TCR transgenic mice were infected intravenously with 200 pfu LCMV and 8 or 13 days after infection spleen cell suspensions were prepared. EL4 (H-2b) target cells were pulsed with LCMV glycoprotein peptide 33-41 at a concentration of 1 µM and labeled with 250 µCi ⁵¹Cr for 90 min at 37°C on a rocking platform. Alternatively, LB27.4 (H-2d/b, MHC class II+) target cells were pulsed
with peptide P13 at a concentration of 100 µM and labeled with 250 µCi $^{51}$Cr. Spleen cells were incubated in 96-well round bottom plates with $10^4$ target cells at effector to target ratios of 90:1, 30:1, 10:1 and 3:1 in 200 µl. After a 5h incubation period at $37^\circ$C, 70 µl of supernatants were harvested and counted.

11. Viral titers
LCMV titers were determined as previously described (Battegay et al., 1991). Organ homogenates were serially 10-fold diluted and incubated in 24-well plates with $2\times10^5$ MC57 cells/well for 48h in a total volume 1.2 ml MEM, 5% FCS at $37^\circ$C. Quantification of viral titers was performed by immuno-staining of intracellular LCMV-NP protein as described previously (Battegay et al., 1991). Titers are shown as log10 pfu per organ.
Vaccinia titers in ovaries were determined 5 days after (challenge) infection as described previously (Binder and Kündig, 1991). Titers are shown as log10 pfu per animal.
Results

1. Generation of transgenic mice expressing a MHC class II-restricted TCR specific for LCMV GP P13
A CD4+ T cell clone (clone1) specific for the LCMV GP-derived I-A^b binding epitope P13 (amino acids GP 61-80) was generated. The peptide P13 epitope represents a LCMV GP-derived I-A^b binding epitope and is of biological relevance since it is presented in vivo by splenic APCs upon LCMV infection and since efficient induction of P13-specific CD4+ T cells is observed after LCMV infection of C57BL/6 mice (Oxenius et al., 1995). The variable regions of the TCR expressed by clone1 are composed of the Vα2.3 and the Vβ8.3 V-elements as defined by FACS staining using the Vα2- and Vβ8.3-specific monoclonal antibodies B20.1 and 7G8 and by sequence analysis of the functionally rearranged variable region genes (data not shown, EMBL nucleotide sequence database accession numbers Y11102 and Y11103).

The genomic Vα2 and Vβ8.3 variable regions genes were cloned into TCR cosmid expression vectors being composed of genomic TCR loci sequences which were previously shown to give rise to efficient expression of rearranged TCR genes (Katz et al., 1993). Coinjection of Vα2 and Vβ8.3 expression vectors into fertilized C57BL/6 eggs and threefold backcrossing of a founder mouse to C57BL/6 mice gave rise to two TCR transgenic mouse lines (Smartal and Smarta2).

2. Transgenic TCR expression on thymocytes
To evaluate thymic selection of the I-AD-restricted transgenic TCR exhibiting specificity for the LCMV GP derived epitope P13, thymocytes of transgenic mice of the selecting H-2^d background or of the non-selecting H-2^d background as well as control thymocytes of negative littermates were subjected to FACS analysis.

Fig.1A presents three-colour cytofluorimetric analysis of thymocytes from Smartal and Smarta2 TCR transgenic mice and from negative littermates that were all on the positively-selecting H-2^b background. Thymocytes numbers of TCR transgenic mice were comparable to negative littermates (data not shown). Smartal and Smarta2 transgenics showed a profound increase in the numbers of CD4^+8^- thymocytes with a concomitant decrease in numbers of CD4^-8^+ thymocytes. This developmental skewing into the CD4 compartment has to be expected since the transgenic TCR originates from a CD4+ T cell clone and since this phenomenon was repeatedly found in TCR transgenic mice (Berg et al., 1989; Foerster et al., 1995; Goverman et al., 1993; Katz et al., 1993; Kaye et al., 1989). A phenotypic difference between the two TCR transgenic mouse lines Smarta1 and Smarta2 is their ability to more or less drastically skew CD4+ T cell development. The CD4:CD8 ratio of single positive
thymocytes is 35:1 in the case of Smarta1; 6:1 in the case of Smarta2. Interestingly, the proportion of CD4⁻8⁻ cells seems to be increased in both TCR transgenic mouse lines as compared to negative littermates whereas the proportion of CD4⁺8⁺ thymocytes is decreased in both TCR transgenic mouse lines as compared to negative littermates.

Both TCR transgenic lines express high levels of the transgene encoded Vβ8.3 and Va2 variable regions on mature CD4⁺ T cells (Fig.1A, lower center and right panels; data only shown for Vβ8.3 expression).

Fig.1B shows three-colour cytofluorimetric analysis of thymocytes originating from TCR transgenic mice on the H-2d genetic background and from control Balb/c mice. Thymocyte numbers of TCR transgenic mice on the non-selecting background were reduced by 50-70% as compared to control mice (data not shown). The CD4⁻8⁻ population is drastically increased in TCR transgenic mice and virtually no single positive thymocytes develop in the thymus. The transgenic TCR chains are expressed on CD4⁻8⁻ and CD4⁺8⁺ thymocytes (data only shown for Vβ8.3 expression).
Fig.1A

Fig. 1B

Fig.1 Cytofluorimetric analysis of thymocytes. In Fig.1A thymocytes from TCR transgenic mouse lines Smarta1 and Smarta2 and from negative littermates were triple stained with tricolour-labeled anti-CD4, FITC-labeled anti-CD8 and phycoerythrin-labeled anti Vα2-specific monoclonal antibodies. Vβ8.3 staining is shown for each CD4+CD8- , CD4+CD8+, CD4+CD8+ and CD4-CD8- thymocyte populations. In Fig.1B identical stainings are shown for TCR transgenic mice on the nonselecting H-2d background and for control Balb/c mice. Representative stainings of four experiments are shown.
3. Transgenic TCR expression on peripheral T cells

In order to characterize peripheral T cell populations in terms of CD4:CD8 ratios and in terms of transgenic TCR expression on CD4+ or CD8+ T cells, three colour cytofluorimetric analysis was performed using mesenterial lymph node cells (Fig.2A) or peripheral blood lymphocytes (Fig.2B) from Smartal, Smarta2 transgenic mice and negative littermates. As already evident from thymocyte analysis, peripheral T cells from TCR transgenics Smartal and Smarta2 revealed a profound skewing of the CD4:CD8 ratio towards the CD4 compartment: The normal CD4:CD8 ratio of 1.8:1 in negative littermates is shifted to a CD4:CD8 ratio of 25:1 in Smartal and 8:1 in Smarta2 (Fig.2A and B, upper rows). Thus, the major phenotypic difference between the two TCR transgenic mouse lines Smartal and Smarta2 is based on a more or less pronounced skewing of the peripheral T cell compartment towards the CD4+ compartment. Transgenic TCR expression on peripheral CD4+ T cells is shown in the lower panels of Fig.2 A and B. More than 95% of peripheral CD4+ T cells express the transgenic TCR in the case of Smartal transgenics and more than 90% in the case of Smarta2 transgenics; normal control mice exhibit only 1-2% of Vα2+Vβ8.3+ peripheral T cells. Interestingly, the transgenic TCR is also expressed on a fraction of CD8+ T cells, a phenomenon that has also been observed in other TCRαβ transgenic mouse lines (Foerster et al., 1995). The presence of such a population may be explained either by the possibility that these CD8+ T cells express a second endogenously rearranged TCR α-chain (Padovan et al., 1993) which enabeled positive selection of a VαxVβ8.3 TCR on MHC class I molecules or alternatively that thymic stochastic lineage commitment allows for the generation of low numbers of CD8+ T cells bearing a MHC class II-restricted TCR (Robey and Fowlkes, 1994). Thus, peripheral CD4+ T cells of Smartal and Smarta2 transgenic mice almost exclusively express the transgene encoded TCR.
Fig. 2

Cytofluorimetric analysis of peripheral T cell populations. In Fig. 2A mesenteric lymph node cells were either double stained with phycoerythrin-labeled anti-CD4 and FITC-labeled anti-CD8 monoclonal antibodies (upper panel) or triple stained with FITC-labeled anti-CD4 (or anti-CD8), phycoerythrin-labeled anti-Vα2 and biotinylated anti-Vβ8.3 monoclonal antibodies followed by incubation with tricolour-conjugated streptavidin (lower panel). In Fig. 2B identical stainings were performed with peripheral blood lymphocytes. Representative stainings of four experiments are shown.
4. Functional assessment of antigen specificity of TCR transgenic CD4+ T cells
To investigate whether or not the transgenic TCR exhibited the appropriate specificity
and whether transgenic CD4+ T cells could be activated by LCMV, in vitro
proliferative responses and concomitant cytokine secretion was assessed (Fig.3 and
4). Splenocytes from naive transgenic animals and from negative littermates were
stimulated with threefold serial dilutions of live LCMV (squares, highest
concentration: MOI=2), with the same amounts of UV-inactivated LCMV (circles),
with the relevant LCMV GP-derived P13 (triangles, highest concentration 0.4 μM) or
with the irrelevant LCMV NP-derived peptide P61 (diamonds, highest concentration
0.4 μM). T cell proliferation was determined by incorporation of 3H-thymidine 3 days
after stimulation (Fig.3). Transgenic T cells were specifically activated by live or UV-
inactivated LCMV, by the LCMV GP-derived peptide P13 but not by the irrelavant
LCMV NP-derived peptide P61. Naive T cells from negative littermates did not
respond to any of these antigens.

Fig.3 In vitro proliferative response of TCR transgenic CD4+ T cells. 6x10^4 naive
TCR transgenic splenocytes or splenocytes from negative littermates were incubated
with 3-fold serial dilutions of either live LCMV (squares), UV-inactivated LCMV
(circles), peptide P13 (triangles) or peptide P61 (diamonds) in the presence of 6x10^5
irradiated C57BL/6 spleen cells. Three days after stimulation T cell proliferation was
determined by incorporation of 3H-thymidine. One of four comparable experiments is
shown.
In addition, cytokine secretion patterns of the above-described in vitro activated transgenic CD4+ T cells were analyzed. Thus, supernatants from activated T cells were collected 24 hrs (IL-2) and 76 hrs (IFNγ and IL-4) after stimulation, 3-fold serially diluted and IL-2, IFNγ and IL-4 contents were determined. IL-2 was assessed using the IL-2-dependent cell line CTLL-2 (Fig.4). Transgenic CD4+ T cells secreted IL-2 upon appropriate stimulation with either live (circles) or UV-inactivated LCMV (squares) or with the relevant peptide P13 (triangles), no IL-2 secretion was detected when stimulated with the irrelevant peptide P61 (diamonds). In contrast, naive T cells from negative littermates did not secrete IL-2 after stimulation with any of these antigens. Similar results were obtained for IFNγ, whereas no IL-4 could be detected in the supernatants of activated transgenic CD4+ T cells (data not shown). These Th1 cytokine patterns are reminiscent of those observed in vivo after LCMV infection (Oxenius et al., 1997a).

Fig.4. In vitro cytokine secretion of activated transgenic T cells. Supernatants of the proliferation assay described in Fig.3 were collected and IL-2 contents in 3-fold serially diluted supernatants were determined using the IL-2 dependent cell line CTLL-2. Viable cells were quantified with the Alamar-Blue® colour reaction assay. Fluorescence emission was assessed at 590 nm. One of four comparable experiments is shown.
Thus, in vitro proliferative responses and concomitant cytokine secretion showed that the TCR transgenic CD4+ T cells are specifically activated upon stimulation with LCMV (live or UV-inactivated) or with the peptide P13.

5. In vivo activation and clonal expansion of transgenic CD4+ T cells

Having established that TCR transgenic T cells responded specifically to LCMV in vitro, it was of interest to determine whether transgenic T cells are activated and clonally expanding in vivo after LCMV infection. Therefore the following adoptive transfer experiments were performed: $10^6$ splenocytes from TCR transgenic mice were adoptively transferred into naive negative littermates which were infected with 200 pfu LCMV either 2 days before transfer (Fig.5, upper panel), at the timepoint of adoptive transfer (Fig.5, middle panel) or 3 days after adoptive transfer (Fig.5, lower panel). Clonal expansion of the transferred transgenic CD4+ T cells was assessed by three colour cytofluorometric analysis of PBLs at the indicated timepoints after adoptive cell transfer (Fig.5, circles). As negative control, $10^6$ transgenic splenocytes were transferred into naive negative littermates without LCMV infection (squares). A pronounced clonal expansion of adoptively transferred transgenic CD4+ T cells was observed in all 3 experimental setups peaking at 40-60% transgenic T cells of total CD4+ PBLs whereas control groups remained at the 1-2% background staining level. The kinetics of expansion upon adoptive transfer was dependent on the viral antigen load present at the timepoint of transfer; recipient mice infected with LCMV 2 days previous to adoptive transfer and thus exhibiting significantly higher viral titers than recipients infected with LCMV at the timepoint of transfer, showed enhanced kinetics of clonal expansion of transgenic CD4+ T cells (upper two panels). Whereas transgenic T cells had already expanded to 40% of total CD4+ PBLs 5 days after adoptive transfer in recipients infected with LCMV 2 days before transfer (upper panel), significant clonal expansion was only observed 7 days after adoptive transfer in recipients infected with LCMV at the timepoint of transfer (middle panel). Clonal expansion of transgenic T cells in recipient mice infected with LCMV only 3 days after transfer showed a delay of 5 days but then was apparent in a sharp peak reaching 60% transgenic T cells of total CD4+ PBLs (lower panel). The constant low background level of CD4+Vα2+Vβ8.3+ PBLs in naive recipient mice which received transgenic CD4+ T cells demonstrated that the observed clonal expansion was LCMV-specific.
Fig. 5 Clonal expansion of adoptively transferred TCR transgenic CD4+ T cells. 1x10^6 naive Smartal TCR transgenic splenocytes were adoptively transferred into negative littermates infected with 200 pfu LCMV either 2 days before transfer (upper panel, circles), at the timepoint of adoptive transfer (middle panel, circles) or 3 days after adoptive transfer (lower panel, circles). As control, the same number of transgenic splenocytes was adoptively transferred into naive negative littermates without LCMV infection (squares). Transgenic CD4+ T cells were assessed by three-colour cytofluorimetric analysis (FITC-labeled anti-CD4, phycoerythrin-labeled anti-Vα2 and biotinylated anti-Vβ8.3 monoclonal antibodies followed by incubation with tricolour-conjugated streptavidin) of PBLs at the indicated timepoints after adoptive transfer. Each line represents one individual recipient mouse. One of three equivalent experiments is shown.
In all cases, the frequency of transgenic CD4+ T cells dropped after the initial expansion to finally reach background levels comparable to naive mice 30 days after adoptive transfer.

6. Antibody responses in TCR transgenic mice
Having demonstrated that the TCR transgenic CD4+ T cells are activated in vivo upon LCMV infection, it was of interest to analyze their functional role in vivo in terms of cognate help for B cells and thus for the production of LCMV-specific antibody responses.

Transgenic mice and negative littermates which were either untreated, anti-CD8 depleted or anti-CD4 depleted were infected with 200 pfu LCMV. 11 days after infection LCMV GP2 and LCMV NP-specific IgG antibody titers were determined by ELISA (Fig.6). Thus, ELISA plates were either coated with recombinant LCMV GP2 or with recombinant LCMV NP. Interestingly, TCR transgenic mice almost exclusively produced LCMV GP2-binding IgG antibodies when untreated and even slightly enhanced titers when anti-CD8 depleted while virtually no LCMV NP-specific IgG response could be detected in all cases (Fig.6 upper panel). CD4-depletion abrogated LCMV-specific IgG production.

In contrast, negative littermates exhibited high titers of LCMV NP-specific IgG antibodies and to a lesser extent LCMV GP2-binding IgG antibodies (lower panel). Again, CD4-depletion inhibited LCMV-specific IgG production. The drastic reduction of LCMV NP-binding IgG antibody production in TCR transgenic mice is apparently a consequence of the strongly biased LCMV GP-specific Th cell repertoire. This result indicates that LCMV NP-specific antibody production strictly requires NP-linked T help. LCMV infection of normal mice leads to the induction of both GP-and NP-specific CD4+ T cells (Oxenius et al., 1995) thus leading to IgG production against both LCMV-derived proteins.
Fig. 6 LCMV-specific IgG antibody responses. Untreated (left panels), CD8-depleted (middle panels) or CD4-depleted (right panels) Smarta TCR transgenic mice (upper panels) or negative littermates (lower panels) were infected with 200 pfu LCMV. 11 days later LCMV GP2-specific (circles) or LCMV NP-specific (squares) IgG titers were determined in 30-fold prediluted sera by ELISA. 3-fold serial dilutions of the sera are shown. Each line represents an individual mouse. One of four equivalent experiments is shown.
Interestingly it has been shown in other experimental viral systems that Th cells specific for virus internal proteins (such as the nucleoprotein (N) or the matrix protein (M) of influenza virus or VSV) are able to provide help for the production of antibodies specific for viral surface antigens; a process which was termed intermolecular/intrastructural help (Mitchison, 1971; Scherle and Gerhard, 1986). Apparently, this is only valid in this one direction since LCMV surface protein (LCMV GP)-specific helper cells are usually not able to provide help for the production of antibodies specific for LCMV internal proteins such as the LCMV NP. The fact that LCMV-specific antibody responses are enhanced after LCMV infection of CD8-depleted mice can be interpreted in two ways: firstly CTL-mediated immunopathology is absent and secondly viral titers and thus antigen load are drastically increased thus efficiently activating a B cell response.

In a second step, the influence of virtually unlimited GP-specific T help in TCR transgenic mice on the production of LCMV neutralizing antibody production was assessed. Interestingly, TCR transgenic animals infected with LCMV did not produce detectable levels of LCMV neutralizing antibodies up to 60 days after infection (data not shown).

Finally the capacity of TCR transgenic CD4+ T cells to provide cognate help for B cell activation and maturation was assessed using a non-infectious, non-LCMV-related B cell epitope. The hapten dinitrophenol (DNP) was chosen as B cell epitope since it is known that DNP-specific B cells usually have a high precursor frequency (Kettman and Dutton, 1970) as compared to virus-specific B cells (Bachmann et al., 1994b; Turtinen et al., 1986). In such a hapten system, T help becomes a limiting factor and it is thus suitable for the direct in vivo assessment of Th cell responsiveness (Charan et al., 1986). Therefore, DNP was covalently coupled to the N-terminus of the LCMV GP-derived peptide P13 which in turn was covalently linked over its C-terminus to BSA (DNP-P13-BSA). The twofold modified P13 was still processed and presented on MHC class II molecules as determined by in vitro activation of a P13-specific CD4+ T cell hybridoma (not shown). TCR transgenic mice, naive C57BL/6 mice or LCMV-immune C57BL/6 mice (50 day previously immunized with LCMV) were immunized intravenously with a limiting amount of DNP-P13-BSA and DNP-specific IgG titers were determined by ELISA 7, 10 and 14 days after immunization (Fig.7). TCR transgenic mice exhibited high titers of DNP-specific IgG antibodies already 7 days after immunization (triangles) whereas naive C57BL/6 mice only showed marginal induction of DNP-specific IgG antibodies (circles). Interestingly, normal LCMV immune mice (squares) showed similar enhancement of DNP-specific IgG production as the naive TCR transgenic mice, indicating that LCMV-specific memory T help may - at least in this experimental setup - be characterized by elevated Th cell precursor frequencies. Similar results
have also been shown for VSV-specific MHC class II-restricted TCRβ transgenic mice (Freer et al., 1995).

Fig. 7 DNP-specific IgG antibody responses. Naive TCR transgenic mice, naive negative littermates or LCMV immune C57BL/6 mice were immunized intravenously with 5 μg DNP-P13-BSA. At the indicated timepoints, DNP-specific IgG titers were determined in 30-fold prediluted sera by ELISA. Each line represents the mean of three individual mice. One of three comparable experiments is shown.

7. Assessment of cytotoxic T cell function in TCR transgenic mice
Perforin-mediated cytotoxicity by LCMV-specific CD8+ T cells has been shown to be instrumental for resolution of LCMV infection (Kägi et al., 1994a). Thus, CTL activity in TCR transgenic mice and in negative littermates was assessed by in vitro 51Cr release assays at different timepoints after infection. Fig. 8 shows CTL activities in the TCR transgenic mouse lines Smartal and Smarta2 and in negative littermates 8 days after infection with 200 pfu LCMV. Specific lysis of EL4 target cells pulsed with the immunodominant LCMV GP-derived Db binding peptide gp33 (upper panel) revealed that the TCR transgenic mouse line Smartal was profoundly impeded in the generation of LCMV-specific CTLs whereas the TCR transgenic mouse line Smarta2 showed reduced but nevertheless significant CTL activity as compared to negative littermates. This finding correlates well with the observation that the skewing of the...
CD4:CD8 ratio is much more pronounced towards the CD4 compartment in Smarta\textsuperscript{1} transgenic mice as compared to Smarta\textsuperscript{2} transgenic mice (Fig.2). Assessment of CTL activity 13 days after infection revealed that at this timepoint also Smarta\textsuperscript{1} transgenic mice showed low levels of CTL mediated specific killing (data not shown).

Fig.8 LCMV-specific cytotoxic activity in vitro. Smarta\textsuperscript{1} (left panels), Smarta\textsuperscript{2} (middle panels) transgenic mice and negative littermates (right panels) were infected with 200 pfu LCMV and CTL activity was determined 8 days after infection by $^{51}$Cr release assay. Target cells were peptide gp33 pulsed EL4 cells (upper panel, squares). Unlabeled EL4 cells (upper panel, circles) served as specificity control. In the lower panel MHC class II positive LB27.4 target cells were used as targets for the same d8 effector cells either labeled with peptide P13 (lower panel, squares) or unlabeled (circles). One of three equivalent experiments is shown.
In an additional experiment, day 8 effector T cells from Smartal and Smarta2 transgenic mice and from negative littermates were assessed for their potential to lyse MHC class II+ LB27.4 target cells (lower panel). LB27.4 target cells were pulsed with the relevant MHC class II binding peptide P13. Neither Smartal nor Smarta2 transgenic mice nor negative littermates showed significant levels of specific lysis of peptide pulsed target cells - although a marked unspecific lysis of target cells was observed in the case of the negative littermates. LB27.4 cells pulsed with the Db binding peptide gp33 were efficiently lysed by d8 effector CTLs from negative littermates (data not shown). These results are in contrast to other findings where ex vivo LCMV-specific MHC class II restricted cytolytic activity was observed in LCMV-infected β2M-deficient mice (Muller et al., 1992).

8. LCMV titers upon infection of TCR transgenic mice
In association with the different levels CTL activities observed in Smartal and Smarta2 TCR transgenic mice, it was of interest to determine the consequences of this finding on viral clearance after infection. Smartal and Smarta2 transgenic mice and negative littermates were infected with 200 pfu LCMV and viral titers were determined in the indicated organs 8 and 14 days after infection (Fig.9). Whereas no LCMV could be detected in any organ at these timepoints in negative littermates (virus could be detected 4 days after infection in the spleen, data not shown), Smartal transgenic mice showed viral titers in all organs at both timepoints. Smarta2 transgenic mice exhibited viral titers in all organs except the blood 8 days after infection; by 14 days after infection virus was cleared from all organs.
Fig. 9 LCMV titers in TCR transgenic mice upon LCMV infection. Smarta1 (left panels) and Smarta2 (middle panels) transgenic mice and negative littermates (right panels) were infected with 200 pfu LCMV and viral titers were determined in indicated organs 8 and 14 days after infection. Each symbol represents an individual mouse. Virus titers (log10) per organ are shown. One of three comparable experiments is shown.
These findings corroborate many earlier observations that CD8+ T cell effector functions are crucial for LCMV clearance during acute infection (Kägi et al., 1994a; Zinkernagel and Welsh, 1976) and that LCMV-specific CD4+ T cells are not capable of directly interfering with LCMV infection. In the situation of the TCR transgenic mice Smartal and Smarta2, LCMV clearance is delayed or even absent due to the skewing of the T cell development into the CD4 compartment leading to a more (Smartal) or less (Smarat2) reduced CD8 T cell compartment in terms of size and/or repertoire. Interestingly, Smartal transgenic mice do not survive low dose LCMV infection but die after 2-3 weeks after infection whereas Smarta2 transgenic mice do survive. The mechanisms responsible for the fatal outcome of infection in Smartal transgenic mice are currently under investigation. These results show that not even extremely high frequencies of LCMV-specific CD4+ T cells are protective against LCMV.

9. Infection of TCR transgenic mice with recombinant vaccinia virus expressing LCMV GP

Having established that CD4+ T cells are not able to directly participate in protective immunity to LCMV, the protective potential of LCMV-specific CD4+ T cells was assessed in another infectious virus model. Protection against recombinant vaccinia virus infection expressing either the VSV glyco- or nucleoprotein was shown to be mediated by either VSV-specific CD4+ T cells or CD8+ T cells depending on the haplotype of the animal (Kündig et al., 1993a). Protection by CD4+ T cells was shown to be mediated by IFNγ and TNFα (Binder and Kündig, 1991; Kündig et al., 1993a). In the case of recombinant vaccinia viruses expressing either the LCMV GP (VV-G2) or LCMV NP (VV-YN4), protection of LCMV immune C57BL/6 mice against challenge infection with these recombinant vaccinia viruses was shown to be mediated by LCMV-specific CD8+ T cells (Dr. T. Kündig, personal communication). It was therefore possible that LCMV-specific CD4+ T cells were not anti-virally protective in general. To test this hypothesis, naive Smarta transgenic mice and and negative littermates were infected with 2x10^6 pfu VV-G2 or VV-YN4 and vaccinia virus titers were determined 5 days later in ovaries (Fig.10). Control mice showed high titers of vaccinia virus after infection with VV-G2 and VV-YN4 (Fig.10, right panel). Interestingly, naive Smartal TCR transgenic mice were protected against infection with VV-G2 expressing the relevant Th cell epitope but not against infection with VV-YN4 expressing an irrelevant Th cell epitope (Fig.10, left panel). Thus, LCMV GP-specific CD4+ T cells show direct and specific protective capacity against infection with recombinant vaccinia virus.
Fig. 10 Vaccinia titers in ovaries of TCR transgenic mice upon infection with recombinant vaccinia virus. TCR transgenic mice (left panel) and negative littermates (right panel) were infected with $2 \times 10^6$ pfu recombinant vaccinia virus either expressing LCMV GP (G2) or expressing LCMV NP (YN4). 5 days after infection vaccinia titers were determined in ovaries. Virus titers (log10) per mouse are shown. One of three equivalent experiments is shown.

Antibody responses are not involved in the protective immune response since recombinantly expressed LCMV GP and NP proteins are not expressed on the surface of the virus. In addition, adoptive transfer of in vitro primed TCR transgenic CD4+ T cells into naive recipients were protective against infection with VV-G2 but not against infection with VV-YN4 (data not shown). This finding is in contrast to the above mentioned observations, namely that normal C57BL/6 LCMV immune mice are protected against challenge infection with VV-G2 or VV-YN4 via LCMV-specific CD8+ T cells. Since LCMV infection of normal C57BL/6 mice leads to a much more efficient activation and clonal expansion of LCMV-specific CD8+ T cells than of LCMV-specific CD4+ T cells, this high number of activated LCMV-specific CD8+ T
cells might positively predispose them to mediate protection upon challenge infection with VV-G2 or VV-YN4. To test this hypothesis, CD8-deficient mice were infected with LCMV with the aim to selectively enhance LCMV-specific CD4+ T cell induction as opposed to the situation in normal mice where LCMV-specific CD8+ T cell induction is dominant. In addition, a group of CD8-deficient mice was depleted of CD4+ T cells. 12 days after LCMV infection, the CD8-deficient mice were challenged with 2x10^6 pfu VV-G2 or VV-YN4. Five days after challenge, vaccinia virus titers were determined in the ovaries (Fig.11). Interestingly, LCMV-infected CD8-deficient mice were protected against challenge infection with VV-G2 or VV-YN4 and this protection was largely dependent on CD4+ T cells since LCMV-infected, CD4-depleted CD8-deficient mice were not protected.

**Vaccinia protection in CD8/−**

![Graph showing vaccinia protection in CD8/− mice](image)

**Fig.11** Vaccinia titers in LCMV infected CD8-deficient mice challenged with recombinant vaccinia virus. Untreated or CD4-depleted CD8-deficient mice were infected with 200 pfu LCMV and were challenged 11 days later with 2x10^6 pfu recombinant vaccinia virus either expressing LCMV GP (VV-G2, left panel) or expressing LCMV NP (VV-YN4, right panel). In addition, naive CD8-deficient mice were infected with the recombinant vaccinia viruses. 5 days after infection vaccinia
titers were determined in ovaries. Virus titers (log10) per mouse ovaries are shown. One of two comparable experiments is shown.

Taken together, these findings indicate that in the case of recombinant vaccinia viruses expressing LCMV-derived proteins (and most probably also for recombinant vaccinia viruses expressing VSV-derived proteins), the T cell compartment involved in protective immunity is selected by the initial T cell priming infection (with LCMV or VSV). Depending on the haplotype and thus on the availability of MHC class I or II-binding epitopes on LCMV or VSV viral proteins and possibly on the TCR repertoire, virus-specific CD4+ or CD8+ T cells are preferentially induced which are able to mediate protection upon challenge infection with recombinant vaccinia viruses. However, if CD8+ T cells specific for a VSV or LCMV epitope are induced in the primary infection (and this can be in addition to CD4+ T cell induction), they seem to be dominant in mediating protection against challenge infection with recombinant vaccinia virus.
Discussion
This study describes a new MHC class II-restricted TCR transgenic mouse with specificity for a viral protein as opposed to most other MHC class II-restricted TCR transgenic mice that exhibit specificity for soluble protein antigens. This new situation allows phenotypical and functional analysis of virus-specific CD4+ T cell responses upon viral infection in vivo. In normal C57BL/6 mice LCMV infection induces CD4+ T cell responses with specificity for the LCMV GP-derived I-A^ binding epitope P13 (amino acids 61-80) and the LCMV NP-derived I-A^ binding epitope P61 (amino acids 309-328) (Oxenius et al., 1995). For the generation of the MHC class II-restricted TCR transgenic mice, a P13-specific CD4+ T cell clone was established whose TCR variable region genes were cloned into genomic TCR expression constructs (Katz et al., 1993). The two TCR transgenic mouse lines Smartal and Smarta2 were characterized phenotypically and functionally upon viral infection. Both TCR transgenic mouse lines positively select the transgenic TCR and express it 90-95% of mature CD4+ T cells and both TCR transgenic mouse lines specifically recognize LCMV in vitro as determined by proliferative responses and cytokine secretion. The transgenic-TCR-induced skewing of T cell development into the CD4 compartment is so pronounced that the CTL response to LCMV is seriously impaired (as discussed later).

1. Functionality of TCR transgenic T cells: antibody response
Analysis of LCMV-specific antibody responses by ELISA in TCR transgenic mice revealed efficient LCMV GP-specific IgG responses which were enhanced as compared to negative littermates. Interestingly, LCMV NP-specific IgG responses were virtually absent in TCR transgenic mice whereas normal mice mounted a strong LCMV NP-specific IgG response which was more pronounced than the LCMV GP-specific IgG response. These results imply that Th cells exhibiting specificity for viral surface antigens are incapable of providing help for virus-internal proteins such as LCMV NP. It has been shown for influenza and VSV infection, that Th cells with specificity for virus-internal proteins (such as N and M) are able to mediate help for B cells with specificity for surface viral proteins (Scherle and Gerhard, 1986; Zinkernagel et al., 1990). This intramolecular help is apparently only valid for Th cells with specificity for virus internal proteins and B cells with specificity for virus external proteins. This suggests that LCMV NP-specific B cells recognize the LCMV NP protein in a physical form where it is no longer structurally linked to the LCMV GP and thus requires intramolecular help from LCMV NP-specific Th cells. NP-specific Th cell responses are induced upon infection of normal mice with LCMV and thus an efficient LCMV NP-specific IgG response can be observed. However, in the case of LCMV GP-specific TCR transgenic mice, the specificity of T help is strongly
biased towards the LCMV glycoprotein resulting in a reduction of LCMV NP-specific antibody production. B cells recognizing viral surface antigens are taking up entire virus particles and are thus able to present any virus protein-derived helper cell epitope on MHC class II molecules; therefore any virus-specific Th cell can provide help to these B cells.

Interestingly, kinetics of LCMV GP-specific IgG production in TCR transgenic mice were not drastically enhanced as compared to normal mice, suggesting that during the normal LCMV-specific antibody response, B cells and not T helper cells are limiting. A similar finding was observed in VSV infection; VSV-specific memory Th cells present at elevated frequencies did not influence the kinetics of VSV-neutralizing IgG responses (Charan et al., 1986). Transgenic mice expressing the Vβ chain of a VSV G-specific MHC class II-restricted TCR showed enhanced efficiency of VSV-neutralizing antibody production upon immunization with a limiting amount of inactivated, non-replicating VSV (Freer et al., 1995) whereas infection with live virus revealed similar kinetics of VSV-neutralizing antibody production in normal and transgenic mice (Dr. Giulia Freer, personal communication). To further analyze this question, the ability of TCR transgenic LCMV GP-specific CD4+ T cells to provide help for B cells present at high precursor frequencies was assessed. Since hapten-specific B cell presursor frequencies are commonly very high (Kettman and Dutton, 1970), a DNP-specific B cell response was analyzed. Thus, a molecule was constructed where DNP was covalently linked to the N-terminus of the LCMV GP-derived helper cell epitope P13 which was covalently coupled to BSA via its C-terminus (DNP-P13-BSA). Immunization of naive TCR transgenic mice with a limiting amount of DNP-P13-BSA induced high DNP-specific IgG titers already 7 days after immunization whereas naive control mice did not mount significant DNP-specific IgG responses. Interestingly, LCMV immune C57BL/6 mice immunized with DNP-P13-BSA showed comparable DNP-specific IgG titers as naive TCR transgenic mice, suggesting that elevated presursor frequencies of specific CD4+ T cells can account for secondary type humoral immune responses under conditions where B cells are not limiting.

Thus, LCMV GP-specific transgenic CD4+ T cells were able to provide cognate help for B cells in vivo. However, LCMV-neutralizing antibody responses (being specific for LCMV GP) could not be detected in TCR transgenic mice upon LCMV infection. In normal mice, neutralizing antibody responses are only detected very late upon infection (>60 days after infection) and several possible explanations might account for this fact. i) LCMV infection induces a vigorous LCMV-specific CTL response which could eliminate LCMV-infected B cells exhibiting LCMV-neutralizing specificity (Planz et al., 1996). ii) The efficient LCMV-specific CTL response induces massive immunopathology in secondary lymphoid organs resulting in generalized
immunosuppression (Odermatt et al., 1991) which in turn deprives B cells of follicular structures which are required for B cell responses. iii) In vivo depletion of CD8+ T cells shifts the kinetics of LCMV-neutralizing antibody responses to earlier timepoints which may be due to lacking immunopathology and/or due to increased viral load an thus increased B cell antigen concentrations. iv) For some reason inefficient T help (possibly due to CTL-mediated immunopathology) might also account for the late appearance of LCMV-neutralizing antibodies. This last point could be excluded in the LCMV GP-specific TCR transgenic mice since LCMV-specific Th cells are present at drastically elevated frequencies, since it has been shown that they are activated upon viral infection and since they have been shown to provide cognate help for B cells. In addition CTL responsiveness in these TCR transgenic mice is reduced as compared to normal control mice, but no enhancement of LCMV-neutralizing antibody response could be observed. Thus, LCMV-specific Th cells do not seem to play an important role for the late appearance of LCMV-neutralizing antibody responses but rather one or more of the other mentioned parameters.

2. Functionality of TCR transgenic T cells: in vivo expansion
A pronounced in vivo clonal expansion of transgenic CD4+ T cells could be observed after adoptive transfer into normal LCMV-infected recipients. The kinetics of clonal expansion was dependent on the amount of antigen present at the timepoint of adoptive transfer. Normal recipients infected with LCMV previously to adoptive cell transfer showed enhanced kinetics of clonal expansion as compared to recipients infected with LCMV at the timepoint of adoptive transfer. After having reached the peak of clonal expansion, transgenic CD4+ T cell numbers slowly returned to background levels 30 days after transfer. This is in contrast to a similar adoptive transfer model described for transgenic, LCMV-specific, MHC class I-restricted CD8+ T cells where transgenic CD8+ T cells remained at elevated frequencies (20-30% transgenic cells of total CD8+ PBLs) for long time periods after clonal expansion (Zimmermann et al., 1996). The former may, however, more closely reflect conventional immune responses, where T cell frequencies decline usually by at least a factor of 100 after the peak of the response.

3. Functionality of TCR transgenic T cells: antiviral protection
LCMV-specific cytotoxic CD8+ T cell responses are delayed and reduced in efficiency in TCR transgenic mice upon LCMV infection. The degree of delay and reduction in efficiency is different in the two TCR transgenic mouse lines Smartal and Smarta2. Smartal and Smarta2 transgenics show a different degree of skewing of T cell development into the CD4 compartment; Smartal transgenics exhibit almost no
mature CD8+ T cells whereas Smarta2 transgenics exhibit significantly more mature CD8+ T cells. This phenotypic difference becomes functionally evident after LCMV infection; while Smarta1 transgenics show moderate levels of MHC class I-restricted killing only late after LCMV infection (>12 days after infection), Smarta2 transgenics exhibit MHC class I-restricted CTL responses already 8 days after infection, although reduced in efficiency as compared to normal control mice. This difference in CTL responsiveness of both TCR transgenic mouse lines is also reflected in LCMV titers detected at different timepoints after infection in several organs. Whereas Smarta1 transgenics showed virus in all organs tested up to 13 days after infection, Smarta2 transgenics are able to clear infection by this timepoint. This corroborates many previous observations that clearance of LCMV infection is crucially dependent on perforin-mediated cytotoxicity by LCMV-specific CD8+ T cells and that LCMV-specific CD4+ effector T cell functions do not directly interfere with LCMV infection and replication (Ahmed et al., 1988; Leist et al., 1987). However, previous studies did not distinguish between inefficient induction of LCMV-specific CD4+ T cells and the presence of LCMV-specific CD4+ T cells that could not interfere with LCMV replication. This study, using a LCMV-specific TCR transgenic mouse model, clearly showed that activated LCMV-specific CD4+ T cells are incapable of directly interfering with LCMV replication and that no specific, MHC class II-restricted cytolytic activity by CD4+ transgenic T cells could be observed after LCMV infection in vivo.

Interestingly, Smarta1 transgenic mice do not survive LCMV infection. It has been shown that infection of normal mice with high doses of widely replicating LCMV leads to exhaustion of virus-specific CTLs and thus to the induction of a life-long virus carrier status (Moskophidis et al., 1993). Therefore it could have been expected that LCMV infection of Smarta1 transgenics almost devoid of CD8+ T cells and leading to uncontrolled virus replication would also induce a virus carrier status. The reason(s) for the fatal outcome of LCMV infection in Smarta1 transgenic mice is currently under investigation; several mechanisms may play a role: In the presence of CD4+ T cells, exhaustion of CD8+ T cells is more difficult to achieve than in the absence of CD4+ T cells (Battegay et al., 1994) which could theoretically result in fatal CD8+ T cell-mediated immunopathology. Alternatively, LCMV-specific CD4+ T cells might directly exhibit immunopathological potential either by the secretion of cytokines or by direct cell-mediated functions.

Since it has been shown that LCMV-specific CD4+ T cells are not capable to directly interfere with noncytopathic LCMV infection, it was of interest to investigate whether these CD4+ T cells showed protective potential against a different viral infection. As opposed to noncytopathic LCMV infection, cytopathic vaccinia virus infection is not controlled by perforin-mediated cytotoxicity (Kägi et al., 1994a). In fact, it has been
shown that either CD4+ or CD8+ T cells can be protective against vaccinia virus infection (Kündig et al., 1993a). This protective effect was shown to be mediated by T cell secreted IFNγ and TNFα (Binder and Kündig, 1991; Kündig et al., 1993a) and under special circumstances these cytokines by their own were protective against vaccinia virus infection: for example it was shown that even immunosuppressed mice infected with recombinant vaccinia viruses expressing either IFNγ or IL-2 were protected against infection (Ramshaw et al., 1992).

To further study the different protective mechanisms for LCMV and vaccinia virus infection, LCMV GP-specific TCR transgenic mice were infected with a recombinant vaccinia virus expressing the LCMV glycoprotein (VV-G2) or with a recombinant vaccinia virus expressing the LCMV NP (VV-YN4) as a specificity control. Interestingly, TCR transgenic mice were protected against infection with VV-G2 but not against infection with VV-YN4, indicating that the LCMV GP-specific TCR transgenic CD4+ T cells were activated by VV-G2 and cleared infection presumably by secretion of anti-virally active cytokines. It is worth mentioning, that normal C57BL/6 LCMV immune mice are protected against challenge infection with recombinant vaccinia viruses expressing LCMV-derived proteins. In this case, however, the protective immunity is mediated by LCMV-specific CD8+ T cells (Dr. T. Kündig, personal communication). Thus, LCMV-specific CD4+ T cells are apparently also capable of mediating protection provided they are present at sufficiently high presursor frequencies. In a normal situation, LCMV infection induces an extremely efficient CTL response which most probably exceeds by far the efficiency of LCMV-specific CD4+ T cell induction. Therefore, the LCMV-specific CD8+ T cells are under normal conditions the prime candidates of mediating protection against challenge infection with recombinant vaccinia viruses expressing LCMV-derived proteins. However, the situation may change i) if in certain mouse strains no CTL epitopes may be present on the recombinant protein of the vaccinia virus (Kündig et al., 1993a) or ii) if a biased T cell population is forming the T cell repertoire. The latter is the case in the LCMV GP-specific TCR transgenic mice. It was additionally of interest whether a CD4+ T cell-mediated anti-vaccinia protection could also be observed in a nontransgenic situation where CD8+ T cell responses are hampered and thus induction of CD4+ T cells is favoured. Therefore, CD8-deficient mice were infected with LCMV and challenged with either VV-G2 or VV-YN4 in the presence or absence of CD4+ T cells. Interestingly, the LCMV primed CD4+ T cells showed protective capacity against recombinant vaccinia virus challenge, indicating that the efficiency by which the priming infection activated CD4+ or CD8+ T cells determined the cell population conferring protective immunity against challenge infection with vaccinia virus. However, if both CD4+ and CD8+ T cells are activated during the primary infection, the CD8+ effector T cells seem to behave in a dominant
fashion in conferring protection upon secondary challenge infection with recombinant vaccinia virus.

Taken together, this new MHC class II-restricted virus-specific TCR transgenic mouse model allows to dissect the involvement of CD4+ T cells in humoral virus-specific immune responses and in cellular immune responses on a clonal level. Depending on the nature of the virus, different effector cell mechanisms are crucially involved in the resolution of infection and CD4+ T cells can directly provide protective immunity in infections which are controlled by the release of anti-virally active cytokines but not in infections which are controlled by direct cell-mediated cytolytic effector functions. Using this TCR transgenic mouse model, it will be of further interest to analyze immunopathological aspects of CD4+ T cell functions, involvement of CD4+ T cells in long term protective immunity and behaviour of CD4+ T cells in chronic virus infections.
3. GENERAL DISCUSSION

CD4+ T cell induction and effector functions: a comparison of immunity against soluble antigens and viral infections

Introduction

The central task of the immune system is to defend the host against a wealth of pathogens in the environment. Studying immune responses to infectious agents such as viruses, bacteria, protozoa and multicellular parasites teaches immunology with respect to its biological purpose and with respect to its evolution which was driven by the coexistence of pathogens and hosts.

Initial immunological research focused on infectious diseases which was followed by a period concentrating on antigen-specific immunity which was based on chemically defined antigens such as soluble proteins (γ-globulin, ovalbumin, lysozyme etc.) or hapten-carrier conjugates. These studies provided insight into the specificity of immune recognition and in the mechanisms of cellular interactions involved in this process; furthermore, these experimental model systems led to the definition of rules governing antigen-specific activation or tolerization of immune responses based on antigen dose, route of application and molecular nature of the antigen. However, generalizations on the basis of some of these results may be difficult because the immune responses elicited by soluble protein model antigens are usually not relevant for the survival of the host (with the exception of toxins and vaccines); kinetics and efficiency of the immune response therefore do not underly the pressure imposed by infectious agents. Nevertheless, based on the conceptual knowledge obtained with purified antigens and thanks to enormous methodological progresses in biochemistry, molecular biology, embryology and cell culture, the key parameters orchestrating an immune response upon infection can and should now be reevaluated in infectious disease models. Certainly, many immunological aspects may be equivalent for soluble model antigens and infectious agents whereas other aspects are unique to infectious disease models: replication kinetics, cell tropism and cytopathogenicity of infectious agents influence time-dependent amplification of the antigen load, geographical localization of the antigen and pathological aspects. In addition, pathogens efficiently trigger responses of the innate immune system, affecting the generation of specific immune responses. The immune response has to cope with the pathogen-induced dynamics by its own dynamics, coordinating its
effector functions in the most efficient way to finally resolve infection or to keep the infection at a minimal level or to even tolerate infection in the case of certain non-cytopathic pathogens. Although the effector mechanisms and effector lymphocytes involved in protective immune responses against different pathogens are usually manyfold and differ in relevance for each pathogen, the following chapters will mainly focus on the role of virus-specific CD4+ T cells. In a first part, parameters influencing Th cell activation after immunization with soluble model antigens in comparison to viral infections will be discussed and in a second part, the role of virus-specific Th cell effector functions in several selected viral disease models will be addressed.

3.1. Activation of CD4+ T cells

3.1.1. Antigen administration, antigen dose and geographical location

Soluble antigens
Soluble antigens able to load MHC class II molecules can be used to activate antigen-specific CD4+ T cells and are usually either whole proteins requiring processing for the presentation of the relevant antigenic peptides on MHC class II molecules or alternatively directly MHC class II-binding antigenic peptides not requiring processing. The routes of administration as well as the dose of soluble antigen have been shown to be a crucial parameter influencing induction of Th cell activation versus Th cell tolerance. The first studies performed in this line demonstrated that very high concentrations of soluble protein administered repeatedly and systemically induced a state of specific immune paralysis (high zone tolerance) which was abrogated after a critical time-threshold, probably when newly generated T and B cells emerged from the thymus or the bone marrow and when the antigen had been eliminated from circulation (Mitchison, 1964; Weigle, 1973). This phenomenon is dependent on the very high doses of antigen and probably leading to a very dense antigen presentation on MHC class II molecules such that virtually every specific Th cell is induced and subsequently repeatedly restimulated, eventually leading to Th cell unresponsiveness and/or deletion. This process of Th cell 'over-activation' has more recently also been demonstrated on the clonal level in vitro using Th cell clones or in vivo using TCR-transgenic animals immunized with high doses of protein antigen or peptide antigen (Critchfield et al., 1994; Kearney et al., 1994; Liblau et al., 1995). Intermediate doses of soluble antigen applied systemically, leading most probably to
a more restricted antigen-presentation pattern, favour proper Th cell activation (Mitchison, 1964). The observation that administration of very low doses of soluble antigen induces Th cell unresponsiveness (Mitchison, 1964) might be explained by the poor antigen presentation of the low concentrations of soluble protein which is most probably restricted to antigen-specific B cells which are the only APCs capable of concentrating antigen via their surface Ig-receptors (Lanzavecchia, 1987). Naive B cells express very little costimulatory molecules and are thus believed to be able to induce Th cell unresponsiveness (Eynon and Parker, 1992; Fuchs and Matzinger, 1992; Gilbert and Weigle, 1994). Thus, Th cell activation seems to critically depend on an appropriate dose of the soluble antigen which preferentially targets the antigen to professional APCs and is generally not easily achieved with truly soluble antigens. In addition, protein aggregates have been shown to induce specific Th cell responses whereas soluble, non-aggregated protein was shown to preferentially induce Th cell tolerance (Weigle, 1973).

A powerful method to improve the antigenicity of soluble proteins or peptides is to administer them in association with adjuvants. Commonly used adjuvants in the murine system are CFA, IFA, alum, liposomes, ISCOM and others. A twofold effect is achieved by administering the soluble antigen in adjuvant: i) The soluble protein is retained within a locally applied antigen-depot, releasing the antigen slowly and over a prolonged time period thus inhibiting rapid consumption and proteolysis of the antigen and enabling a prolonged presentation of the relevant antigen on MHC class II molecules on APCs. ii) The addition of heat killed mycobacteria (such as in CFA) and LPS induces local inflammation which leads to non-antigen mediated upregulation of costimulatory molecules on the APC surface enhancing the quality of the APC for activation of naive Th cells.

The localization of the administered antigen depends on the route of application and/or the use of adjuvants retaining the antigen at a certain location; systemic immunization with soluble antigens leads to rapid dissemination over the blood and thereby to dilution of the antigen; in contrast, local application in adjuvants directs the antigen primarily to the local draining lymph node. Systemically applied soluble antigen is most probably only poorly filtered out of the circulation in the spleen by the phagocytosing marginal zone macrophages which are responsible for trapping particulate antigens; thus, antigen capture is most probably performed by antigen-specific B cells which are able to concentrate antigen via their surface Ig-receptors. On the other hand, locally administered antigen can be efficiently trapped by immature dendritic cells (DC) (such as the Langerhans cells in the skin) via macropinocytosis or mannose receptor-mediated uptake and is then transported within the DC to the local draining lymph node where it is presented to the pool of recirculating naive Th cells (Sallusto and Lanzavecchia, 1994). However, a
characteristic common to all soluble antigens, independent of their route of administration, is their quantitatively defined concentration which never increases after application but rather diminishes over time due to proteolysis and consumption. Taken together, soluble antigens such as proteins or peptides are usually poor activators of naive Th cells unless administered together with adjuvants (Warren et al., 1986). In fact, systemically administered soluble antigens usually even lead to induction of Th cell unresponsiveness. It is interesting to note that soluble proteins administered systemically may induce tolerance even independently of the antigen dose. Many proteins used for the above mentioned studies were presumably contaminated with LPS which has the ability to activate macrophages and B cells. At very low and at very high concentrations, LPS would either be too diluted or its effect overwhelmed by the protein and Th cells would be tolerized, while at intermediate doses, LPS would unspecifically activate macrophages and B cells and thus prevent Th cell tolerance induction. In this scenario, Th cell priming at intermediate protein doses would represent in fact a LPS-mediated artefact (W. O. Weigle, pers. communication).

Infectious agents
The major differences between soluble non-replicating antigens and infectious replicating agents are i) the dynamics of antigen concentration due to amplification of the antigen, ii) the antigen localization which is not only directed by the route of infection but also by the cell tropism of the virus, replication kinetics and spread within the host. Thus, the antigen dose actually present at a given site of the organism at a given timepoint after infection is very difficult to measure and thus only a limited quantitative control can be exerted. The limiting and augmenting factors concerning antigen concentration are manyfold and interrelated: virus replication kinetics, cytopathogenicity leading to liberation of newly synthesized antigens or immunopathology caused by immune effector cells all contribute to an increasing antigen load. On the other hand, immune mechanisms such as innate immunity and specific T and B cell responses reduce the antigen load by clearing virus. In addition, the number of antigen-presenting cells is influenced by viral cytopathogenicity or immunopathology. Thus, antigen concentrations start usually at a defined concentration of the inoculum, amplify to certain levels and drop later on, when infection is controlled by the immune system. A probably crucial difference between viral antigens and soluble antigens is, that the former are always locally very highly concentrated. The local antigen concentrations may in fact exceed by far what is usually achieved upon immunization with exogenous soluble proteins (Battegay et al., 1996).
Depending on the cell tropism and the route of infection, virus-derived antigens first appear in distinct secondary lymphoid organs: systemic application of viruses leads to capture of the virus by the marginal zone macrophages of the spleen; local administration of the virus leads either to local replication in suitable host cells and thus finally to the liberation of viral antigens which can be either trapped by locally resident immature DC or macrophages or can be directly transported to the draining lymph node via the lymph. Infection via mucosal surfaces usually leads to antigen presentation in Peyers patches or in the tonsils (Mims, 1987; Paul, 1993). As for the soluble model antigens, activation of virus-specific naive Th cells occurs in secondary lymphoid organs where epitopes of virus-derived proteins are presented on MHC class II molecules.

Despite the difficult quantification of antigen load upon viral infections, the initial virus dose in the inoculum as well as the replication kinetics of the virus have been shown to influence the functional outcome of T cell activation: In the case of CD8+ T cells it has been demonstrated for example that infection with high doses of widely replicating LCMV-Docile leads to exhaustion of virus-specific CTLs and thus to the establishment of a virus carrier status (Moskophidis et al., 1993). Such an exhaustive activation of virus-specific CD4+ T cells has not yet been described after overwhelming virus infection.

3.1.2. Antigen presentation on MHC class II molecules

**Soluble antigens**

In contrast to MHC class I molecules which are (or can be) expressed on virtually all nucleated cells within an organism, MHC class II molecules are usually only expressed on antigen presenting cells such as dendritic cells, macrophages and B cells. APCs are - via different mechanisms - capable of trapping soluble antigens, antigen complexes or particulate antigens which are subsequently degraded in specialized intracellular compartments to peptide fragments which are potentially able to load MHC class II molecules. The complex of MHC class II and peptide antigen reaches then the cell surface where it can be recognized by antigen-specific Th cells (Germain and Margulies, 1993). The different mechanisms of antigen uptake involve i) phagocytosis of particulate antigen or antigen complexes by macrophages, ii) macropinocytosis or mannose receptor-mediated uptake of antigen by immature dendritic cells, iii) Ig receptor-mediated uptake by antigen-specific B cells and iv) Fc-receptor-mediated uptake of antibody-antigen complexes mainly by macrophages. In all cases the antigen is trapped after uptake in endosomal vesicles which are
progressively acidified, thus enabling acidic proteases to continuously degrade antigen into peptide fragments (Neefjes and Momburg, 1993). Heterodimeric MHC class II molecules - consisting of an α and β chain - are newly synthesized in the ER where they associate with the invariant chain (Ii) (Cresswell, 1992). The association with Ii is thought to i) prevent (at least to some extent) loading of newly synthesized MHC class II molecules with ER-resident peptides, ii) traffic newly synthesized MHC class II molecules to endosomal compartments due to an Ii-inherent endosomal targeting signal (Bakke and Dobberstein, 1990; Lamb et al., 1991) and iii) to thermodynamically stabilize the structure of the MHC class II αβ heterodimer (Sant and Miller, 1994; Stockinger et al., 1989). MHC class II molecules are then transported to endosomal compartments where they are loaded primarily, but not exclusively (Castellino and Germain, 1995), with antigenic peptides within the recently described MIIC compartment (Amigorena et al., 1994; Tulp et al., 1994). Having reached the endosomal compartments, the still associated Ii is subjected to proteolysis (above all by cathepsin S (Riese et al., 1996)) resulting finally in MHC class II αβ heterodimers being loaded with a residual 20-25-mer Ii-fragment (CLIP, class II associated invariant chain peptide) (Ghosh et al., 1995). The CLIP fragment is exchanged with the antigenic peptides by the catalytic action of the MHC class II locus encoded H2-M molecule (HLA-DM is the corresponding molecule in humans) (Denzin and Cresswell, 1995; Miyazaki et al., 1996; Sherman et al., 1995). Ultimately, the MHC class II-peptide complexes are shuttled to the cell surface where they can be recognized by CD4+ T cells. The functional importance of Ii and H2-M molecules in the above described MHC class II loading pathway for soluble antigens has recently been documented by the virtual absent capacity of Ii- or H2-M-deficient APCs to utilize soluble protein antigens for MHC class II presentation in vitro. MHC class II molecules from Ii-deficient mice were shown to be mostly SDS-instable (Miller and Germain, 1986; Viville et al., 1993) whereas MHC class II molecules from H2-M deficient mice were shown to almost exclusively be loaded with the CLIP peptide (Fung-Leung et al., 1996; Miyazaki et al., 1996).

In contrast to soluble antigens which require antigen processing, MHC class II binding peptides do not require processing and are thus able to load any MHC class II expressing APC - including the above mentioned Ii- and H2-M-deficient APCs. MHC class II binding peptides may either bind to empty MHC class II molecules which are newly synthesized or are recycling from the cell surface to endosomal compartments, or class II binding peptides may be taken up during the process of endocytosis/macropinocytosis to be loaded onto MHC class II molecules in endosomal compartments.
Infectious agents

In addition to the above described 'classical' MHC class II presentation pathway, viruses may use additional pathways for MHC class II loading. One major difference to exogenous, soluble proteins is, that intracellular pathogens such as viruses synthesize viral proteins within the infected cell. These intracellularly synthesized proteins are usually the sources of MHC class I binding peptides but may also - in certain situations - be able to load MHC class II molecules. Intracellularly synthesized viral proteins are a priori not different from any intracellularly synthesized host cell protein and thus studies, investigating the mechanism(s) of MHC class II presentation of endogenously synthesized host cell proteins, may be equally informative for MHC class II presentation of endogenously synthesized viral antigens. Many studies on the mechanism of MHC class II presentation of endogenously synthesized proteins have revealed apparently contradictory results. MHC class II presentation studies performed with intracellularly synthesized self or neo-self antigens in professional antigen presenting cells almost exclusively demonstrated, that certain proteins naturally residing in or passing through the ER during their biosynthesis were able to load MHC class II molecules (Bikoff et al., 1995; Bodmer et al., 1994; Chen et al., 1990; Moreno et al., 1991; Newcomb and Cresswell, 1993; Rudensky et al., 1991a; Rudensky et al., 1991b; Weiss and Bogen, 1991). In contrast, endogenously synthesized proteins which do not have natural access to the ER are generally not found to be presented on MHC class II molecules. The pathways used by the intracellularly synthesized proteins are apparently not identical in all cases, since in some studies the presence of Ii proved to be inhibitory for MHC class II loading (Bodmer et al., 1994; Newcomb and Cresswell, 1993) whereas in other studies the presence of Ii seemed to be irrelevant for MHC class II loading (Malnati et al., 1992; Nuchtern et al., 1989; Sweetser et al., 1989). Endogenously synthesized membrane proteins might alternatively load MHC class II molecules during a membrane-endosome recycling process which would predictably involve membrane protein processing in endosomal compartments. Although this might be true for some membrane proteins, it has also been demonstrated that certain membrane proteins do not require acidic proteolysis - thus favouring a more direct MHC class II loading mechanism (Chen et al., 1990).

Several studies on MHC class II presentation of infectious virus-derived endogenously synthesized antigens are not easily generalizable. The difficulty in comparing these results are based on the fact that often non professional APCs were used for these studies. The relevance of the nature of the APC has been revealed for example by studies where identical intracellularly synthesized antigens were differentially capable of loading MHC class II molecules in fibroblasts as compared to myeloma cells (Bikoff, 1991; Bikoff, 1992; Moreno et al., 1991). In addition and
most importantly, the characteristics of a viral infection in terms of cytopathogenicity, influence on host cell membrane structure and host cell protein synthesis and processing may lead to contradictory experimental findings. Several studies performed with recombinant vaccinia viruses revealed that MHC class II loading by intracellularly synthesized viral proteins was generally chloroquine-sensitive - regardless of the subcellular localization of the recombinant proteins (Jaraquemada et al., 1990; Jin et al., 1988; Malnati et al., 1992). The fact, that vaccinia infection leads to a major shut-down in cellular host protein synthesis resembling treatment with BFA or cycloheximide (Fields, 1990) may deprive the infected APC of newly synthesized MHC class II molecules which could potentially be loaded outside endosomal compartments. Thus, only recycled MHC class II molecules from the surface would be available for loading by recycled virus-derived membrane proteins, a process which should be inhibitable by chloroquine (Polydefkis et al., 1990). In addition, cytoplasmic proteins derived from recombinant vaccinia viruses appear to gain access to endosomal compartments possibly by degradation-promoting pathways (Ciechanover, 1994), by association with heat shock proteins (Schirmbeck and Reimann, 1994) or as a result of the large intracellular aggregates formed by vaccinia that might fuse with endosomal compartments (Fields, 1990). Recently it has been shown that the half life of a cytosolic protein is an important factor determining its ability to load MHC class II molecules; the longer the half life the better the chances of MHC class II loading (Guéguen and Long, 1996). Since vaccinia virus and influenza virus are both cytopathic viruses, it is conceivable that they disrupt intracellular compartments. This renders a comparison of the processing and antigen presentation pathways of viral proteins in different subcellular localizations difficult - if not impossible - since the proteins may finally be uniformly distributed within the virus-infected cell.

Thus, infectious viral systems using non-cytopathic viruses such as lymphocytic choriomeningitis virus (LCMV) that only minimally affect host cell functions represent convenient systems to study antigen presentation of endogenously synthesized viral proteins (Oxenius et al., 1995). Analysis of MHC class II presentation of endogenously synthesized viral proteins after LCMV infection of professional APCs revealed that the subcellular localization of the protein crucially influences whether or not MHC class II loading can occur independently of acidic compartments. In analogy to the data obtained with transfected cell lines and by elution and analysis of natural self-peptides bound to MHC class II molecules, this study demonstrated, that only intracellularly synthesized membrane-associated LCMV-glycoprotein was able to load MHC class II molecules independently of processing in acidic compartments, whereas the intracellularly synthesized cytosolic LCMV-nucleoprotein was shown to be incapable of loading MHC class II molecules
in professional APCs. In addition, this process was shown to be TAP-independent, suggesting that the intracellularly synthesized LCMV-GP, reaching the ER upon its natural biosynthetic pathway, is most probably able to bind to newly synthesized MHC class II molecules by efficiently competing with Ii.

In general, these data indicate that alternative - albeit less efficient - processing pathways exist, allowing MHC class II molecules to be loaded with endogenously synthesized proteins. In the case of viral infections with non-cytopathic viruses, virus-infected professional APCs may be able to activate virus-specific Th cells even before the infected cell is lysed and before viral antigens are liberated to enter the 'classical' MHC class II presentation pathway.

Under normal circumstances the assessment of such alternative MHC class II presentation pathways in vivo has proven to be difficult, mainly because of the dominating efficiency of the classical presentation pathway. Nevertheless, under circumstances where classical antigen presentation is severely hampered, functional MHC class II presentation by endogenously synthesized viral proteins could be demonstrated after LCMV infection of Ii-deficient mice. At early timepoints after infection, only endogenously synthesized membrane-associated LCMV-glycoprotein and not cytosolic LCMV nucleoprotein was presented on professional APCs leading to early, selective activation of LCMV-glycoprotein-specific Th cells (Oxenius et al., 1997a). Whereas replication-deficient LCMV was unable to induce LCMV-specific IgG antibodies, infection with replicating LCMV induced a slightly delayed but significant anti-LCMV IgG response. This suggests that non-cytopathic viruses infecting APCs and being capable of presenting Th cell epitopes derived from endogenously synthesized proteins are able to functionally induce Th cells more readily than only after CD8+ T cell mediated cell lysis which would liberate sufficiently enough viral antigens which could enter the 'classical' MHC class II presentation pathway.

### 3.1.3. APC targeting

**Soluble antigens**

Administration of soluble antigen in vivo leading to MHC class II associated antigen presentation has revealed apparently conflicting results in terms of the APC subset primarily targeted by a certain antigen (Constant et al., 1995b; Constant et al., 1995c; Guéry et al., 1996; Levin et al., 1993). The form in which an antigen is delivered (soluble protein antigen in saline, soluble protein antigen in adjuvant, peptide antigens) may critically favour the targeting of one APC subset or the other. In several studies it has been demonstrated that administration of peptide antigen in
adjuvant requires the presence of dendritic cells for priming of naïve CD4+ T cells whereas the protein form of the same antigen is presented only poorly by dendritic cells (Constant et al., 1995b; Constant et al., 1995c; Levin et al., 1993). Instead, the presence of B cells seems to be a prerequisite for priming with protein antigen (Constant et al., 1995b)- especially if the antigen concentration is limiting. Contradictory results were obtained by Guéry et al. who showed that dendritic cells but not B cells present antigenic complexes to CD4+ T cells following administration of protein antigen in adjuvant (Guéry et al., 1996). The requirement of B cells to present protein antigen to CD4+ T cells apparently varies from one protein to another; while HEL, KLH and OVA do not seem to absolutely require B cells for activation of naïve CD4+ T cells, cytochrome c, chicken γ-globulin, conalbumin and human collagen IV seem to require B cells as APCs for priming CD4+ T cell responses (Constant et al., 1995c). Several considerations may help to explain - at least partly - these contradictory observations: i) the dose of antigen effectively present upon in vivo administration (influenced by the stability of the protein); preferential targeting of the protein to antigen-specific B cells might occur if the antigen concentrations are limiting since it has been shown that antigen-specific B cells are able to efficiently concentrate and process protein antigen by means of their surface Ig receptors (Lanzavecchia, 1987). ii) The precursor frequency of antigen-specific B cells may influence their involvement in protein antigen presentation, i.e. B cells at a very low precursor frequency or exhibiting low avidities to the protein antigen might not be able to reach a critical threshold concentration of APC density or antigen density to induce priming of naïve CD4+ T cells. iii) The form of the antigen effectively present upon administration might play a role: mixing of protein antigens with adjuvant such as CFA or IFA leads to the denaturation of the proteins. Denatured proteins have often the tendency to aggregate - especially if natively hydrophobic core domains are exposed to each other upon denaturation. Thus, protein aggregates might be preferentially phagocytosed by macrophages or macropinocytosed by immature dendritic cells. iv) most read-out systems defining the APC primarily involved in antigen presentation were indirect read-outs, i.e. the capacity of a certain APC subset to activate naïve CD4+ T cells in vivo. The activation of naïve CD4+ T cells requires distinct costimulatory signals provided by the APC in addition to MHC class II presentation of the relevant epitope. These costimulatory requirements might thus play an important - if not the crucial - role for the differential APC requirements of certain soluble antigens for priming of naïve CD4+ T cells. This issue will be addressed in more detail in the next chapter.
**Infectious agents**

The targeting of different subsets of APCs by antigens derived from infectious viruses might be divided into two categories: i) The cell tropism of the virus, replication kinetics, cytopathogenicity and immunopathology influence geographical antigen distribution, antigen load and the integrity of antigen presenting cell compartments in secondary lymphoid organs whereas ii) virus-derived antigens liberated from infected host cells may be physically regarded as equivalent to soluble protein antigens and thus some of the above mentioned parameters for APC targeting might as well be applied to these viral antigens - although viral antigens most probably never appear in truly soluble form but are usually aggregated and/or membrane associated. The cell tropism of a virus may influence either in a positive and or a negative fashion the kinetics and the efficiency by which viral determinants are presented on MHC class II molecules on professional APCs. On one side, viruses infecting directly professional APCs such as LCMV, EBV and HIV potentially can very rapidly present viral determinants on MHC class II molecules by class II loading with intracellularly synthesized viral proteins (Oxenius et al., 1995) and thus rapidly activate naive CD4+ T cells. But efficient and prolonged presentation is only achieved if the virus is not rapidly cytopathic thus destroying the APC within a short time period upon infection or if the infected APC is not readily lysed by cytotoxic effector T cells which can result in severe immunopathology accompanied with immunosuppression (Odermatt et al., 1991). On the other hand, viruses not infecting professional APCs or viruses which cannot load MHC class II molecules by intracellularly synthesized proteins require host cell destruction and liberation of viral antigens which can be taken up by professional APCs. Depending on the cytopathic or non-cytopathic character of the viral infection, infected cells are either lysed by the virus or by CD8+ effector T cells. The thus liberated antigens most probably reach high local concentrations (Battegay et al., 1996) sufficient to over-ride APC deficiencies (such as li-deficiency) and thus are able to load MHC class II molecules on local APCs. The aggregated or membrane-associated physical appearance of viral protein antigens derived from lysed virus-infected cells are prone to phagocytosis. In addition, since these protein-aggregates exhibit usually native protein structures, antigen-specific B cells might also - at least to some extent - be involved in antigen uptake and processing. Especially due to the postulated multimeric appearance of these antigens, the Ig receptors on the surface of virus-specific B cells are likely to be cross-linked resulting in upregulation of costimulatory molecules (Cassel and Schwartz, 1994) and thus most probably enabling priming of naive CD4+ T cells and facilitating T-B collaboration.

Finally, certain viruses escape immune recognition by i) latency, ii) in the case of retroviruses by silent integration into the host cell genome or iii) lack or mutation of
MHC-binding epitopes. Such escape mechanisms apparently lead to either failure of MHC presentation of virus-derived determinants or to ignorance of the presented epitopes by the Th cells.

3.1.4. Th cell activation

**Soluble antigens**

CD4+ T cells recognize antigenic peptides bound to MHC class II molecules on the surface of APCs via their T cell receptor. This interaction confers the specificity component for the interaction of a given Th cell with peptide-loaded MHC class II molecules and is - in terms of Th cell activation - generally described as signal 1. This signal 1 alone has many times been shown to be insufficient for activation of naive Th cells (Dubey et al., 1995; Jenkins, 1994). More precisely, it has been shown to induce Th cell unresponsiveness (anergy) rather than Th cell activation (Celis and Saibara, 1992; Harding et al., 1992; Schwartz, 1990). Thus, the activation of antigen-specific naive CD4+ T cells requires a second signaling event which must be equally provided by the APC (Mueller et al., 1989). This second signal is provided by costimulatory molecules located on the APC surface, the best characterized being the B7 molecules (B7-1 and B7-2) that have been shown to act as ligands for CD28 on resting CD4+ T cells (Linsley and Ledbetter, 1993). Recently, the interaction of CD40 on the APC (especially on B cells) and CD40L (expressed on activated CD4+ T cells) has been suggested to be of costimulatory character (Grewal et al., 1995; van Essen et al., 1995). But since CD40L is only expressed on activated CD4+ T cells, this CD40-CD40L interaction is apparently not a costimulatory signal for the activation of naive CD4+ T cells but might be - in certain experimental systems - important for the clonal expansion of antigen-specific Th cells (van Essen et al., 1995). This, however remains to be shown and the apparent requirement of CD40-CD40L interaction for the clonal expansion of pigeon-cytochrome c-specific CD4+ T cells probably reflects the requirement of CD40-competent B cells for Th cell priming (Constant et al., 1995c) rather than a general requirement of CD40-CD40L interaction for Th cell induction.

In the section of this chapter on infectious agents, the issue of whether CD40-CD40L interactions are crucially involved in Th cell activation following viral infection (Oxenius et al., 1996) will be discussed.

The requirement for the two types of signals to successfully activate naive CD4+ T cells has led to much controversy regarding the putative role of different classes of professional APCs in CD4+ T cell priming *in vivo*. Based on their constitutive expression of costimulatory molecules as well as their abundance of surface MHC class II molecules, it has been postulated that the dendritic cell is the only subset of
APCs having the capacity to prime naive CD4+ T cells (Cassel and Schwartz, 1994; Steinmann, 1991). Indeed, the potency of dendritic cells to activate resting CD4+ T cells either in vitro (Cassel and Schwartz, 1994; Ellis et al., 1991; Inaba and Steinmann, 1985) or in vivo (Inaba et al., 1990; Levin et al., 1993; Sornasse et al., 1992) is well documented. Macrophages and B cells, on the other hand, have been shown to require activation signals themselves to up-regulate costimulatory molecules and to become competent APCs for resting T cells (Janeway and Bottomly, 1994; Jenkins, 1994). Such activation signals include soluble factors such as IFNγ, IFNα, bacterial carbohydrates or other microbial constituents in the case of macrophages and IFNγ, microbial polysaccharides or surface-Ig cross-linking antigens in the case of B cells. Nevertheless, mature dendritic cells also have passed through an activation phase: immature dendritic cells located at potential entry sites of pathogens such as the Langerhans cells in the skin, are very efficient in antigen-uptake and it is only upon GM-CSF and IL-4-induced activation that they up-regulate MHC and costimulatory molecule expression to become fully competent dendritic cells after migration into local secondary lymphoid organs (Sallusto and Lanzavecchia, 1994). However, there still exists an unresolved controversy on the issue of whether B cells play a central role in the initiation of Th cell responses. Several groups have reported that mice deprived of B cells were unable to be primed by soluble antigens for proliferative T cell responses, suggesting that B cells are essential for CD4+ T cell priming (Constant et al., 1995b; Janeway et al., 1987; Kurt-Jones et al., 1988; Ron et al., 1981; Ron and Sprent, 1987). This failure to prime was restored when purified antigen-specific B cells were transferred into the B cell-deficient mice (Kurt-Jones et al., 1988; Ron and Sprent, 1987). In contrast, other studies indicated that B cells were not necessary for the priming of CD4+ T cell responses (Bottomly et al., 1980; Gotoff, 1968; Lassila et al., 1988; Ron and Sprent, 1987; Ronchese and Hausmann, 1993; Sunshine et al., 1991). Even studies using the same experimental antigen such as KLH revealed different results: Epstein et al. reported successful T cell priming in B cell-deficient mice (Epstein et al., 1995) whereas Liu et al. failed to obtain T cell priming (Liu et al., 1995). In addition, several groups reported that antigen presentation by B cells is rather tolerogenic than immunogenic for naive T cells (Eynon and Parker, 1992; Fuchs and Matzinger, 1992; Gilbert and Weigle, 1994). Taken together, the relevance of B cells in the activation/tolerization of naive Th cells may depend on several factors such as i) the ability of an antigen to induce costimulatory molecule expression on B cells (e.g. by cross-linking of the Ig receptors) or via a certain cytokine milieu, ii) the actual concentration of the antigen (low concentrations requiring antigen concentration via the Ig receptors of B cells), iii) the physical appearance of the antigen (truly soluble proteins versus aggregates) and iv) the precursor frequency of antigen-specific B cells.
Another aspect of Th cell activation which recently has been increasingly a focus of interest, is the description of apparently discrete Th cell phenotypes as defined by distinct cytokine secretion patterns. A cellular basis of this distinctive pattern of lymphokine production was provided by the seminal observation of Mosman and Coffman, that long term clones of mouse CD4+ T cells could be subdivided into those producing IL-2, IFNγ and TNFα (Th1 clones) and those producing IL-4, IL-5, IL-6, IL-10 and IL-13 (Th2 clones) (Mosmann and Coffman, 1987; Mosmann and Coffman, 1989b; Mosmann and Coffmann, 1989). In many studies the selective generation of Th1 and Th2 CD4+ T cell clones was performed by the addition of either IL-12 or IFNγ (Th1 clones) or IL-4 (Th2 clones) into the culture medium (Mosmann and Coffman, 1989b; O’Garra et al., 1993; O’Garra and Murphy, 1994; Swain et al., 1990). Long-term Th1 or Th2 clones have lost their reversibility and seem to be definitively polarized (Murphy et al., 1996) and the production of the discrete cytokine patterns has been analyzed on a single cell level using intracellular FACS staining (Assenmacher et al., 1994). Functional aspects of the discrete Th cell populations will be discussed in chapter 2.

The possible factors governing the acquisition of a certain Th cell phenotype have been the subject of many investigations. The parameters suggested to influence Th1/Th2 development involve i) the subset of the professional APC (Duncan and Swain, 1994), ii) the cytokine milieu during the process of Th cell priming (Swain et al., 1991), iii) the antigen dose (Hosken et al., 1995), iv) inherent characteristics of an antigen (Julia et al., 1996), v) the genetic background of the animal (Hsieh et al., 1995) and vi) the degree of TCR cross-linking (Constant et al., 1995a). The relevance of these parameters for the selective induction of Th1 or Th2 phenotypes in murine Th cells in vivo is still a matter of debate and most probably it is the synergism of different parameters that will determine the phenotype of the activated Th cell. A second open question is whether or not activation of Th cells in vivo leads to the generation of such distinct polarized Th cell populations as demonstrated for Th cell clones in vitro or whether the situation in vivo might rather move within a continuum between the two extremes.

In general, appropriate Th cell activation leads to a series of intracellular signal transduction events which originate at the TCR-CD3 complex and finally lead to the induction of transcription of certain genes, the entry of the cell into the cell cycle resulting in clonal expansion, up- or down-regulation of certain surface molecules such as CD69, CD40L, IL-2R or L-selectin and the acquisition of effector functions such as the capacity to secrete certain cytokines and the capacity to provide cognate help for B cell activation. Effector functions of Th cells will be described in more detail in chapter 2.
Infectious agents

Since MHC class II presentation usually occurs after uptake of soluble proteins / protein complexes, an implication of this mode of antigen presentation is, that the direct interaction of CD4+ T cells with virally infected cells is not required for CD4+ T cell activation - as opposed to CD8+ T cells which are preferentially activated by virus infected cells. However, some viruses such as measles virus, HIV-1 and LCMV have tropisms for cells bearing MHC class II molecules. In this circumstance, CD4+ T cells may recognize and be directly activated by the infected cell due to the ability of some endogenously synthesized proteins to directly load MHC class II molecules (Oxenius et al., 1995; Oxenius et al., 1997a) (Long, 1992; Malnati et al., 1992; Pinet et al., 1994).

In addition to the above discussed requirements and mechanisms leading to activation of naive CD4+ T cells (such as the necessity of costimulatory molecule expression on APCs and the parameters influencing the induction of Th1 versus Th2 Th cell phenotypes), several additional aspects influencing and governing Th cell activation upon infection have to be considered: viral infection of the host organism leads to the induction of multiple immune effector functions aiming to defend the organism against the virus. Activated innate defence mechanisms involve the activation of IFNγ-producing NK cells which leads to macrophage activation and thus the upregulation of costimulatory molecule expression. Several microbial constituents have been shown to directly induce costimulatory molecule expression on macrophages probably by the binding to certain receptors recognizing microbial constituents. It seems likely that these receptors originally evolved to allow the phagocytic cells in primitive organisms to recognize microorganisms by binding to structures such as bacterial carbohydrates or lipopolysaccharides that were not found in eukaryotes. These receptors still serve this function in innate immunity as well as playing an important part in the initiation of adaptive immune responses. The induction of costimulatory molecule expression by common microbial constituents is believed to allow the immune system to discriminate between antigens borne by infectious agents and antigens associated with innocuous proteins including self proteins (if not administered in adjuvant) (Janeway jr., 1989; Liu and Janeway Jr., 1991; Razi-Wolf et al., 1992).

Usually viral infections lead to local inflammatory reactions at the site of infection attracting several pro-inflammatory cells such as polymorphonuclear leukocytes, monocytes and macrophages. Upon inflammation, a local cytokine milieu is achieved which is again favourable for the activation of APCs such as for example for locally resident immature dendritic cells. Besides dendritic cells and macrophages also B cells are usually rapidly activated to express costimulatory molecules since viral particles always express surface antigens in a more or less highly repetitive form and
thus induce cross-linking of the surface Ig-receptors on a virus-specific B cell. The cross-linking is sufficient to up-regulate costimulatory molecule expression on B cells (June et al., 1994) and may allow for Th cell activation by B cells. These considerations together with the dynamic kinetics of the viral antigenic burden (influenced by replication kinetics and host cell destruction) and the fact that viral infections usually induce a complex immune response exhibiting innate immune effector functions, cytotoxic T cell activation and antibody production might all be responsible for less strictly defined Th cell activation pathways after infection as compared to the probably more subtle pathways described for protein antigens. Recently, the CD40-CD40L interaction between APCs and activated Th cells has been shown to i) exhibit costimulatory function in experimental systems using soluble proteins (Grewal et al., 1995; van Essen et al., 1995) and ii) to govern protective Th1 phenotype Th cell development in certain infectious experimental systems such as infection with Leishmania (Campbell et al., 1996; Kamanaka et al., 1996; Soong et al., 1996; Stüber et al., 1996). The primary defect in CD40- or CD40L-deficient mice upon Leishmania infection was demonstrated to be a lack of IL-12 production by macrophages which is apparently instrumental in resolving Leishmania infection. This lack of IL-12 production was apparently causing an impairment of macrophages to reach a leishmaniocidal activation state. In contrast, Th cell induction and effector functions upon viral infections seemed to be less dependent on a functional CD40-CD40L interaction: VSV infection in the absence of CD40-CD40L interaction induced Th cell effectors which were able to protect against a challenge infection with a recombinant vaccinia virus expressing the VSV-G. In addition, LCMV infection of CD40-deficient mice induced a Th1-type Th cell response which was able to mediate cognate help to CD40-competent B cells upon adoptive transfer. Furthermore, the induction of LCMV-specific Th cell responses was shown to occur independently of B cells potentially serving as APCs (offering the possibility to analyze involvement of CD40-CD40L interaction in Th cell priming apart from B cell APC involvement), thus rendering dendritic cells or macrophages the prominent APC subsets in Th cell induction upon LCMV infection (Oxenius et al., 1996). These observations strongly suggest that activation of at least certain anti-viral Th cell responses is not critically dependent on CD40-CD40L costimulatory interaction whereas protein antigens are more dependent and anti-Leishmania protective immune responses seem to critically depend on a certain activation status of 'effector' macrophages which is apparently not achieved in the absence of Th1-type effector Th cells as observed in CD40- or CD40L-deficient mice. On the other hand, not only some viral infections were shown to functionally induce virus-specific Th cells in the absence of a CD40-CD40L interaction, but also immunization with sheep red blood cells (SRBC) induced normal Th cell responses in the absence of CD40-CD40L interaction (Foy et al., 1993). As
discussed above, the observed impairment of Th cell responses in the absence of functional CD40-CD40L interaction may be due to the absence of activated B cells or more generally due to the absence of costimulatory competent APCs in the case of immunizations with soluble antigens. This view is supported by the findings i) that adoptive transfer of B7.1-transfected APCs into CD40L-deficient mice can rescue the Th cell response (Grewal et al., 1996) and ii) that CD40-dependent upregulation of B7.2 in vivo lead to full reconstitution of cellular and humoral immune responses (Yang and Wilson, 1996).

The phenotype of Th cells induced upon viral infection seems to be generally skewed into the Th1 phenotype direction. This is reflected by i) the predominance of IL-2- and IFNγ-producing virus-specific Th cells and ii) the predominant IgG2a isotype of anti-viral antibodies (Coutelier et al., 1987; Zinkemagel, 1993). In addition, intracellular bacteria such as listeria and mycobacteria induce Th1-dominated Th cell responses (Abbas et al., 1996; Hsieh et al., 1993). Whether this biased Th1 phenotype development is influenced by innate immune mechanisms associated with a certain cytokine milieu induced after viral infection, by the antigen concentrations reached after virus infection, by the antigen-targeted APCs or whether some inherent characteristics of viral epitopes are involved in this phenotype commitment is still unclear.

On the other hand, protozoa infections such as Leishmania have been shown to be able to induce both Th1 and Th2-type Th cell responses depending on the genetic background of the animal (Boom et al., 1990; Heinzel et al., 1989) and on the Leishmania T cell epitope controlling initial Th cell induction: recently it has been demonstrated that the normally observed non-protective Th2 Th cell induction in BALB/c mice upon Leishmania infection was crucially controlled by the immunodominant LACK-epitope (Leishmania homolog of receptors for activated C kinase) during initial Th cell activation. If this epitope was functionally deleted or if the respective Th cells were tolerized, Th1 Th cell development was observed upon Leishmania infection in BALB/c mice (Julia et al., 1996).

Taken together, the parameters governing Th1 and Th2 phenotype development upon infection are still poorly defined and it seems likely that besides the genetic background of a host the infectious agent may selectively influence the phenotype of Th cell responses. It will be of great interest and value to define these parameters in more detail to understand on one hand the mechanisms pathogens apply to drive Th cell phenotype development and on the other hand to be able to efficiently interfere with these mechanisms in order to manipulate Th cell responses in favour of the host.
3.2. CD4+ T cell effector functions

Effector functions mediated by activated CD4+ T cells include i) cognate help for B cells promoting B cell activation and Ig isotype switch, ii) secretion of certain cytokines exhibiting multiple functions such as optimal activation of different cell subsets (macrophages, dendritic cells, cytotoxic T cells etc.), direct effects on pathogen replication/survival and governing isotype production by activated B cells, iii) delayed type hypersensitivity reactions and iv) certain CD4+ T cells have been shown to exhibit cytolytic potential. In general, CD4+ T cells are believed to play a central role in the regulation of the cooperation of the different arms of an immune response in acute and especially in chronic infections. The relevance of CD4+ effector T cell functions in viral infections differ from one virus to another and have therefore to be characterized for each viral infection separately.

Initial studies characterizing Th cell effector functions in vivo were performed by using soluble and chemically defined antigens for immunization. In vitro parameters characterizing antigen-specific Th cell activation are recall Th cell proliferations and assessment of concomitant cytokine release. In vivo parameters, which are biologically more relevant for the analysis of Th cell effector functions, include the generation of (isotype-switched) antigen-specific antibody responses as well as antigen-specific DTH reactions upon (re)immunization with soluble antigens. Since both antibody production and DTH reaction are also Th cell effector functions in infectious model systems, this chapter will focus on the different Th cell effector functions and their relevance in the control of different viral infections. In a first part, a more general overview about different Th cell effector functions in infectious model systems will be given and in a second part, several selected infectious model systems will be shortly discussed in terms of CD4+ effector T cell functions involved or dispensable for effective resolution of infection.

One of the most important and thoroughly studied effector mechanisms of virus-specific Th cell is their capacity to drive the production of virus-specific antibodies. Neutralizing anti-viral antibodies (with specificity for viral surface antigens) have been shown to be crucially involved in the resolution of certain viral infections (e.g. VSV, rabies (Lefrancois, 1984; Murphy, 1977; Wagner, 1987)). Antibodies represent the most efficient defence against secondary infections with the same virus (Mims, 1987; Thomsen and Marker, 1988). Certain viruses such as VSV as well as some repetitive soluble antigens are able to activate specific B cells independently of T help (TI-antigens) and they are usually characterized by a highly repetitive organization of the relevant B cell antigen either on the surface of a virus or as an inherent structure of the antigen. TI-antigen-mediated activation of antigen-specific B cells results in the
secretion of IgM isotype antibodies but induction of isotype switch is strictly dependent on specific T help (Bachmann and Zinkernagel, 1996).

Several studies have established that Th cells can support antigen-specific B cell responses through two distinct pathways (reviewed in (Melchers and Andersson, 1984) and (Julius, 1982)). One, referred to as cognate help, is thought to result from direct T-B interaction, while the other, referred to as non-cognate or bystander help, is mediated through factors released from activated T cells that act in a non-cognate fashion on antigen-activated B cells. The former is most probably more relevant for antigen-specific B cells responses. Classic cognate help appears to require covalently linked T and B cell determinants whereas special rules may apply, however, for T-B interactions in antiviral B cell responses. For example, mice primed with influenza virus cores (devoid of the surface antigens hemagglutinin, HA and neuraminidase, NA) or with purified matrix (M) protein exhibited an enhanced anti-HA antibody response when challenged with intact virus, but not when challenged with a mixture of virus cores and purified HA (Russell, 1979; Russell and Liew, 1980). Since viral internal determinants are not covalently linked to HA, the observed 'cognate' help was termed intermolecular/intrastructural help (Lake and Mitchison, 1976). Comparable findings were reported using Th cell clones specific for internal influenza virus proteins which could mediate help for anti-HA antibody production (Scherle and Gerhard, 1986) or VSV-induced autoantibody production in transgenic mice expressing the VSV-G (Zinkernagel et al., 1990). On the other hand, Th cells with specificity for viral surface antigens are unable to mediate help for B cells specific for virus internal proteins, demonstrating that intramolecular help is required to promote antibody responses against virus internal proteins. Apparently, virus internal proteins are at the timepoint of binding to slgs on B cells no longer physically linked to viral surface antigens (Oxenius et al., 1997b).

Surface molecules critically involved in successful cognate T-B interaction leading to B cell activation involve TCR-MHC-peptide interaction and interaction between CD40L on the activated Th cell and CD40 on the virus-specific B cell. The relevance of a functional CD40-CD40L interaction in terms of antibody production has been both demonstrated for soluble antigens (Banchereau et al., 1994; Parker, 1993) and for virus infections (Borrow et al., 1996; Oxenius et al., 1996).

The kinetics of antiviral antibody production seems to depend more on the availability and precursor frequency of B cells exhibiting antiviral specificity than on the presence of activated T help since Th cell priming previous to virus infection often only marginally affects the kinetics of antiviral antibody production. This has for example been demonstrated for the kinetics of VSV-neutralizing antibody production (Charan et al., 1986), for LCMV-binding antibody production (unpublished results) and for the late appearance of HIV-neutralizing antibodies and
of LCMV-neutralizing antibodies where virus-specific T help is primed at early
timepoints after infection (Oxenius et al., 1995; Pantaleo and Fauci, 1995).

Another important feature of Th cell effector functions is the secretion of various
cytokines which can exert their function locally and systemically such as direct
inhibition of virus replication and the amplification and regulation of other
components of the host immune response. The cytokine pattern secreted by certain Th
cells has often been correlated with distinct Th1 or Th2 phenotypes of activated Th
cells. Although this strict classification into Th1 and Th2 subsets has been manyfold
clearly demonstrated in *in vitro* studies and also in a few *in vivo* infectious model
systems such as Leishmania infection, it is still a matter of debate whether or not such
clearly defined Th cell phenotypes are generally induced upon infection or whether
they represent a rather extreme situation. Nevertheless, distinct effector functions can
be attributed to different T cell secreted cytokines. The Th1-type cytokines include
IL-2, IFNγ and TNF which are all involved preferentially in cell mediated immune
responses such as DTH reactions and cytotoxic T cell responses. IL-2 is the major
growth factor for antigen-specific CD8+ and CD4+ T cells, it activates NK cells and
promotes B cell differentiation (Farrar et al., 1982). IFNγ and TNF activate
macrophages and NK cells, inhibit replication of some viruses directly and promote
resistance of uninfected cells to viral infection (Staeheli, 1990; Wong and Goeddel,
1986). It should be noted though, that IFNγ is also produced by NK cells (Handa et
al., 1983). In addition to IFNγ produced by T and NK cells, IFNβ is produced by
fibroblasts and IFNα by leukocytes at the site of infection. Virus-derived double-
stranded RNA for example is a potent inducer of interferons. The type of interferon
interfering with viral replication is dependent on the virus and the infected cell type.
For example, vaccinia virus was shown to be most sensitive to inhibition by IFNγ,
less by IFNα and least of all by IFNβ (Meshkova and Mentkevich, 1987; Metz and
Esteban, 1972). Inhibition of translation and/or degradation of viral mRNAs can
account for many aspects of interferon-mediated inhibition of virus replication but
does not rule out other mechanisms (Kerr and Brown, 1978; Paez and Esteban, 1984;
Rice and Kerr, 1984).

Th2-type cytokines include IL-4, IL-5 and IL-10 which are involved in promoting
humoral responses and differentiation of eosinophils and mast cells (Chen and

The paracrine effects of cytokines secreted by CD4+ T cells can be locally focussed
by cell-cell interactions: cognate interaction of T and B cells for example leads to
polar release of the cytokines at the site of cell contact, thus specifically promoting
activation and differentiation of the virus-specific B cell involved in cognate
interaction (Kupfer et al., 1991; Lanzavecchia, 1990).
The distinctive cytokine profiles attributed to Th1 and Th2 Th cell subsets may play a critical role in the control of pathogens if CD4+ Th cell responses become strongly skewed into one or the other subset during infection. As mentioned above, this was initially appreciated in vivo for parasitic infections such as Leishmaniasis: acquisition of a Th1 phenotype by CD4+ T cells leads to resolution of infection whereas acquisition of a Th2 phenotype leads to disease progression (Heinzel et al., 1989). The opposite was shown for infection with Trichuris muris (Else et al., 1994). These examples demonstrate, that distinct Th cell phenotypes may be critically involved in several infectious model systems. It has been suggested that Th1-like Th cell responses are especially beneficial for the resolution of infections with obligate intracellular pathogens such as viruses, since they have been shown to promote cell-mediated responses and local inflammatory responses. Indeed, most virus infections induce Th1-type cytokine secreting Th cells; at least during acute phases of infection. Cytokine profiles secreted by murine CD4+ T cells obtained from lymph nodes and bronchoalveolar lavage fluid upon acute nonfatal infection with influenza virus were strongly biased towards the Th1 pattern (Carding et al., 1993; Sarawar and Doherty, 1994). Furthermore, Th cell responses after acute infection with LCMV were shown to be dominated by IFNγ- and IL-2-producing LCMV-specific Th cells (Oxenius et al., 1996). Human CD4+ T cells specific for HIV-1 have also been shown - at least early upon infection - to exhibit a Th1-type cytokine secretion pattern. In some reports, however, it was shown to change to a Th2-type pattern at later stages of infection (Clerici et al., 1993; Clerici and Shearer, 1994).

In addition, adoptive transfer experiments with polarized virus-specific Th cell clones have provided information about antiviral and biological activities of individual Th cell subsets. For example adoptive transfer of polarized Th1 cells specific for influenza virus mediated protection against influenza virus whereas adoptive transfer of polarized Th2 cells failed to promote recovery from infection (Graham et al., 1994). A preponderance of Th1 or Th2 cytokine profiles during infection might result from an initially 'uncommitted' CD4+ T cell that acquires a certain phenotype during the course of the infection and/or Th cells exhibiting a certain phenotype may selectively overgrow others. In addition, a positive feedback mechanism exerted by the phenotype of committed Th1 or Th2 cells may act to skew the phenotype of the Th cell response further into Th1 or Th2 character. Thus, early events of the immune response such as innate immunity dominated by IFNγ or early preferential IL-4 production might be crucial in directing the phenotype of the Th cell response induced.

Selective expression or neutralization of Th1-type or Th2-type cytokines at the sites of viral infection have also provided information about their immunoregulatory
character and their direct antiviral effects: For example infections with recombinant vaccinia virus expressing either IL-2, IFNγ, TNF or IL-4 have clearly demonstrated that the Th1-type cytokines IL-2, IFNγ and TNF were able to resolve the infection - even in T cell-deficient mice - whereas IL-4 expression at the site of infection inhibited resolution of infection (Ramsay et al., 1993; Ramshaw et al., 1992).

Delayed type hypersensitivity reactions (DTH) are a further effect of certain Th1-type cytokines secreted by CD4+ T cells at the site of infection: CD4+ T cells secrete intercrines (MIF, MCF), IFNγ which activate local macrophages thus increasing the release of inflammatory mediators, TNF-β which causes local tissue destruction and leads to increased expression of adhesion molecules on local blood vessels and IL-3/GM-CSF which activates monocyte production by bone marrow stem cells (Cher and Mosmann, 1987).

A fourth effector mechanism of CD4+ T cells has been claimed to be a direct cytolytic activity against antigen-loaded target cells; some virus-specific CD4+ T cells have been shown to lyse specifically MHC class II+ virus-infected target cells in vitro and thus - potentially - could directly eliminate virus-infected cells in vivo (Fleischer, 1984; Koelle et al., 1994; Muller et al., 1992). However, the relevance of these in vitro findings for in vivo situations still remains unclear, especially i) since most viruses infect a much broader range of target cells in vivo than only MHC class II+ cells and ii) since virus-infected MHC class II+ cells always concomitantly express MHC class I molecules, thus they should be targets for virus-specific CD8+ effector CTLs. The large body of evidence for functional cytotoxicity of CD8+ T cells in vivo suggests that this population is much more important for the lysis of virus-infected cells and thus the direct cytotoxic activity of CD4+ T cells is unlikely to be an obligate in vivo effector function (Kägi et al., 1996).

A fifth effector mechanism which may be viewed as a regulatory mechanism of CD4+ T cells consists of T help for optimal CTL induction. Although it has been demonstrated in various viral infectious model systems that CD8+ effector CTLs can be obtained without the presence of CD4+ T cells such as for example for LCMV (Ahmed et al., 1988; Leist et al., 1989b), ectromelia (Buller et al., 1987), CMV (Koszinowski, 1991), vaccinia (Binder and Kündig, 1991), HBV (Ando et al., 1993) and influenza (Tripp et al., 1995), the magnitude of the CTL response was usually reduced in the absence of T help (Leist et al., 1989b). Nevertheless, in these situations CD8+ T cells were induced efficiently enough to control acute viral infection, although different viruses may more or less depended on the presence of CD4+ T cells for the induction of efficient CTL responses. The cell tropism of a virus (infection of professional APCs or not) may play an important role for the requirement of T help for efficient CTL induction: Viruses infecting professional APCs (e.g. LCMV or ectromelia) and thus readily and optimally activating naïve
CD8+ T cells to become effector CTLs are much less dependent or even independent on T help than viruses infecting other cell subsets. It is interesting to note, however, that sustained CTL responsiveness might in both cases be dependent on T help, since CTL responses in chronic viral infections (Matloubian et al., 1994) seem to depend on T help as well as CTL responses induced upon infection with large doses of widely replicating viruses which are prone exhaustion (Battegay et al., 1994; Moskophidis et al., 1993). The mechanism by which CD4+ T cells mediate help for CTLs is still not clearly defined but it most probably involves Th cell secreted IL-2. In contrast to cognate T-B interactions, T-T interactions are certainly not of cognate nature (at least in the mouse) since murine T cells do not express MHC class II molecules.

The relevance of virus-specific Th cell responses for the resolution of different viral infections cannot be generally formulated but has to be investigated for each virus independently. Using adoptive transfer experiments of purified lymphocyte populations or using in vivo depletion techniques with monoclonal 'therapeutic' antibodies have provided information about the protective role and the relevance of different cell subsets in different infectious viral model systems. More recently, thanks to the development of transgene and gene 'knock-out' technologies in the murine system, mice exhibiting permanent deficiencies of selected cell populations could be generated (e.g. no mature CD4+ T cells are present in MHC class II-deficient mice and no mature CD8+ T cells are present in β2M-deficient mice) or on the other hand, usually extremely heterogenous cell populations (such as T and B cells) could be selectively enriched in desired (virus-specific) specificities by the transgene expression of an appropriate receptor (e.g. TCR or immunoglobulin). The latter also allows the observation of virus-specific cells upon infection in vivo on a cellular level. The results concerning the protective involvement of CD4+ T cells in several viral infections are apparently controversial, but the different parameters often applied in these studies such as virus dose and/or virulence of a virus strain as well as short-term versus long-term protective read-outs might explain some of these discrepancies. In addition, in the case of genetically modified mice, the immune system may develop some degree of flexibility to compensate for a deficiency which is present during ontogeny, thus rendering comparisons with normal mice sometimes difficult. In the next paragraphs the involvement of virus-specific CD4+ T cell responses in protective immunity against some selected viral infections will be shortly summarized.
Lymphocytic choriomeningitis virus (LCMV)

LCMV is a noncytopathic arenavirus whose natural host is the mouse. A large body of evidence demonstrates that protective immunity is primarily mediated by LCMV-specific CD8+ effector T cells after acute systemic infection (Buchmeier et al., 1980; Kägi et al., 1994a; Moskophidis et al., 1987; Zinkernagel and Althage, 1977). Depletion of CD4+ T cells by monoclonal antibodies only reduces CTL activity by a factor of 5-15 which does not influence the resolution of acute infection (Ahmed et al., 1988; Leist et al., 1989b). Similarly cytotoxic T cell responses were efficiently induced in MHC class II-deficient mice capable of eliminating LCMV after acute infection (Battegay et al., 1996). Nevertheless, CD4+ T cells were shown to be required to sustain CD8+ T cell responses during chronic LCMV infection: Whereas normal mice were able to clear certain LCMV isolates within 3 months after infection, mice even transiently depleted of CD4+ T cells became life long carriers and this was correlated with a disappearing CTL response (Matloubian et al., 1994). Thus, CD4+ T cell function may become critical if CTL activity has to be maintained for several weeks or months. This may be relevant for HIV infection where antiviral CD8+ T cell responses are ultimately lost maybe due to loss of T help. Also in this line, Battegay et al. demonstrated that adult CD4+ T cell-deficient mice exhibited enhanced establishment of a virus carrier status upon infection with widely replicating LCMV-Docile (Battegay et al., 1994).

CD4+ T cells have also been demonstrated to be involved in the establishment of optimal CTL memory in terms of CTL precursor frequencies: CD4-depleted mice showed reduced precursor frequencies of memory CTLs and were less protected against challenge infection than untreated control mice (von Herrath et al., 1996). A direct protective role of a LCMV-specific Th cell clone against LCMV infection has been demonstrated by La Posta et al.: Adoptive transfer of a LCMV glycoprotein-specific Th cell clone mediated protection against low doses of intracerebrally inoculated LCMV-Armstrong virus (La Posta et al., 1993). Most probably Th cell clone secreted cytokines such as IFNγ are able to control or at least delay low dose LCMV infection in secluded compartments such as the brain, where diffusion of cytokines is minimal due to the blood-brain barrier (Kündig et al., 1993).

The generation of a LCMV-specific antibody response is strictly dependent on T help (Ahmed et al., 1988). Although LCMV-neutralizing antibodies only appear very late after initial infection and in moderate titers (Battegay et al., 1993), antiviral antibodies seem to play a crucial role in protection against reinfection (Thomsen and Marker, 1988). Thus preexisting neutralizing anti-LCMV antibody titers efficiently inhibited infection with LCMV (Dr. Peter Seiler, personal communication) and even LCMV immune serum with little or no neutralizing activity was shown to markedly reduce both virus spread and replication as well as LCMV-specific cellular immune
responses (Thomsen and Marker, 1988). On the other hand, in certain situations, neutralizing antibodies have also been demonstrated to enhance LCMV-induced disease in the absence of primed cytotoxic T cell responses (Battegay et al., 1993). Preexisting neutralizing antibodies showed little effect on local virus spread in peripheral tissues but they reduced hematogenic spread and infection of antigen presenting cells, thus delaying the generation of primary cytotoxic T cell responses and indirectly modulating the extent of immunopathology in peripheral organs. Thus, neutralizing antibody responses may modulate the CTL response in a beneficial or harmful way depending on i) preexisting antibody titers, ii) the site of infection and iii) the replicative kinetics and the cell tropism of the virus strain.

In addition, recent studies showed that CD4-mediated LCMV-specific antibody production might play an important role in the long term control of LCMV infection. MHC class II-deficient mice as well as B cell-deficient mice were perfectly able to control acute LCMV infection, however, viral titers spontaneously reappeared within 2-3 months after infection and no CTL memory could be demonstrated (Thomsen et al., 1996). This indicates that long term control of LCMV infection is dependent on all three major components of the immune system whereas control of acute infection is primarily mediated by CD8+ effector T cells.

As described for some vaccinia and influenza virus-specific CD4+ T cell clones in vitro, Muller et al. reported on the appearance of LCMV-specific CD4+ 'cytotoxic' T cells upon immunization of β2M-deficient mice with LCMV (Muller et al., 1992). Although cytolytic potential of LCMV-specific CD4+ T cells could be shown in vitro, the question whether or not this cytolytic function plays any role in vivo remains at least doubtful since, despite the presence of these cytolytic CD4+ T cells, LCMV could not be efficiently controlled in β2M-deficient mice after infection nor in CD8-deficient mice (Fung-Leung et al., 1991a). In addition, it is conceivable that these cytolytic CD4+ T cells are only generated and observable in mice which are permanently deficient of CD8+ cytotoxic T cells and may thus be the result of the plasticity of the immune system to compensate for lacking certain effector functions - especially since cytolytic CD4+ T cells were never detected upon LCMV infection of normal mice. In addition, LCMV infection of MHC class II-restricted TCR transgenic mice with specificity for a LCMV GP epitope revealed that LCMV-specific CD4+ T cells are incapable of controlling LCMV infection - even if present at extremely high numbers (Oxenius et al., 1997b).

**Human immunodeficiency virus (HIV)**

The pathological changes of the immune system observed after HIV infection and especially the associated decline of CD4+ T cell numbers and loss of CD4+ T cell function has offered direct evidence for the important role of CD4+ T cells in...
successful host immune responses to viral (and other) infections in humans. HIV-specific CD4+ T cells are usually difficult to recover from HIV-infected individuals. This is probably due to i) infection of large numbers of HIV-specific CD4+ T cells in lymph nodes, since infectious HIV particles were shown to be trapped in the form of immune complexes on follicular dendritic cells and thus activation of HIV-specific Th cells by HIV-presenting APCs in the lymph node is likely to result in intimate contact with infectious virus (Pantaleo et al., 1994). These infected HIV-specific Th cells are one hand targets for CTL-mediated lysis as well as impaired in their function and in their survival since the half-life of an HIV-infected Th cell is around one and a half days (Ho, 1995; Perelson et al., 1996) ii) Chronic (over)stimulation of HIV-specific CD4+ T cells in secondary lymphoid organs (due to the antigen reservoir in follicular structures) might lead to exhaustive activation as has been described for CD8+ T cells during chronic LCMV infection (Moskophidis et al., 1993). HIV-infected individuals commonly exhibit first a loss of HIV-specific CD4+ T cell responses before a more general dysfunction of CD4+ T cells is observed (Clerici et al., 1994). This may contribute to the eventual inability of the host to control HIV infection in a manner comparable to the failure of CD4-deficient mice to control infection with intermediate doses of widely replicating LCMV-Docile (Battegay et al., 1994). Thus, CD4+ T cells might be important for the sustained maintenance of CTL activity over a long time period (Matloubian et al., 1994). Another important parameter possibly leading to the disappearance of CD4+ T cell responses as well as finally to the disappearance of CD8+ T cell responses might be the complete destruction of lymphoid tissue as disease progresses (Pantaleo et al., 1993).

Some investigators suggested a critical role of the Th1/Th2 Th cell phenotype balance for the course of HIV infection. Th1-type Th cell responsiveness was primarily found in long term non progressors and was correlated with an efficient cellular immune response. On the other hand, Th2-type Th cell responsiveness was suggested to contribute to promote disease progression to AIDS (Clerici and Shearer, 1994). In vitro, it has been demonstrated that Th1 clones are less efficiently infected with HIV as compared to Th2 clones (Romagnani et al., 1994). Th2-type cytokines increase activation induced programmed cell death in PBMCs of HIV-infected individuals whereas Th1-type cytokines can prevent such activation induced programmed cell death which may also partly account for the decline of CD4+ T cell counts at late stages of disease (Clerici and Shearer, 1994).

In addition to contributions of CD4+ T cells in the control of HIV infection, patients with reduced CD4+ T cell counts and/or functions show enhanced susceptibility to opportunistic infections and tumors, demonstrating that CD4+ T cell functions are critically involved in many viral, bacterial, mycobacterial, fungal and protozoal infections.
**Hepatitis B virus (HBV)**

A variety of possible outcomes are associated with infection of noncytopathic, hepatotropic hepatitis B virus. The infection may be acute, associated with hepatitis of a few weeks of duration, or persistent, either without disease, or associated with chronic liver disease and hepatoma formation. The immune response to HBV-encoded antigens is responsible for both viral clearance and for disease pathogenesis during HBV infection. The humoral response against viral env-antigens contributes to the elimination of circulating virus particles; the cellular response which is directed against env-, nucleocapsid- and pol-antigens eliminates infected cells. Both MHC class I- and MHC class II-restricted responses are vigorous in acutely infected patients who clear the virus but these responses are relatively weak in chronically infected patients (Chisari, 1995). During acute infection usually a strong anti-nucleocapsid antigen-specific Th cell response is observed and only a weak anti-env antigen-specific Th cell response. Since anti-env responses can clearly be induced after vaccination with plasma-derived or recombinant HBsAg (Celis et al., 1984; Celis et al., 1988) and since anti-env Th cell responses may occur in preclinical incubation periods of disease (Vento et al., 1987), it has been suggested that HBV-env-specific Th cells may be exhaustively activated by high doses of virus and/or by high concentrations of circulating HBsAg that may occur if the early immune response is incapable of rapidly and completely eradicating infection. The nucleocapsid-specific Th cell response temporally coincides with viral clearance in patients with acute hepatitis and thus has been attributed a critical role in viral clearance (Ferrari et al., 1990) - especially since chronically infected patients usually only show weak nucleocapsid-specific Th cell responses. (Ferrari et al., 1990). The functional significance of a vigorous nucleocapsid-specific Th cell response may include a contribution for HBV-specific CTL activation (Missale et al., 1993; Nayersina et al., 1993; Penna et al., 1991) and intermolecular help for antibody production against HBV surface antigens (i.e. HBV neutralizing antibodies) (Milich and McLachlan, 1986; Milich et al., 1987).

Intrahepatic macrophages express MHC-class II molecules and they can efficiently process and present HBV antigens. In chronic disease, mostly Th1-type Th cells are found in the liver (Barnaba et al., 1994) which are able to release inflammatory cytokines to recruit antigen-non-specific inflammatory cells that amplify the pathologic effect mediated by CD8+ effector T cells. On the other hand, cytokines such as IFNγ, TNFα and IL-2 have been shown to act beneficially by noncytolytically inhibiting HBV gene expression and viral replication, thus interfering with the viral life cycle and possibly contributing to viral clearance without destruction of the infected cell (Chisari, 1995).
Influenza

Influenza virus is a virus commonly used to study host-virus relationships. Influenza virus is a negative stranded RNA virus whose genome consists of 8 single stranded segments which encode at least 10 polypeptides including hemagglutinin (HA), neuraminidase (NA), nucleoprotein, (NP) and matrix protein (M) (Choppin and Compans, 1975). Both cellular and humoral immune responses are involved in successful clearance of infection (Virelizier et al., 1979). HA-specific antibodies have been shown to neutralize influenza virus and play a major role for protection against subsequent infection (Virelizier, 1975). On the other hand, cytotoxic T cells have been shown to be able to clear the virus during ongoing infection (Ennis et al., 1978; Yap et al., 1978). Both the humoral and to a lesser extent the cytotoxic T cell response appear to be Th cell-dependent (Askonas et al., 1981; Burns et al., 1975). Hence, the activation of influenza virus-specific Th cells may influence the magnitude and efficiency of the anti-influenza immune response.

Concerning the prominent phenotype of Th cells induced after influenza infection, different observations were reported: in situ mRNA hybridization studies during primary and secondary murine influenza pneumonia revealed that IFNγ and TNFβ were predominantly produced by CD8+ T cells whereas IL-4 and IL-10 were predominantly produced by CD4+ T cells. Interestingly, the frequency of cytokine producing lymphocytes early in mesenterial lymph nodes and late in the lung was much higher than the assumed precursor frequency of virus-specific effectors. If this may be generalized, induction of cytokine gene expression for T cells that are not responding directly to the invading pathogen may be a prominent feature of virus infections (Carding et al., 1993; Tough et al., 1996). On the other hand - focussing on the protective potential of influenza-specific Th cells - it has been reported that adoptive transfer of influenza-specific Th2 clones (in vitro non-cytolytic) did not promote recovery from experimental influenza infection but rather exacerbated pulmonary pathology with concomitant eosinophilia. Adoptive transfer of influenza-specific Th1 clones (in vitro cytolytic) was protective in vivo after virus challenge (Graham et al., 1994). A more indirect protective capacity of influenza-specific Th cell clones was reported by Scherle et al., who demonstrated that mice can recover from pulmonary influenza virus infection in the absence of class I-restricted CTLs. Intranasal exposure of T cell-deficient nu/nu mice resulted in persistent infection followed by death after 3-4 weeks. Adoptive transfer of CD4+ T cell clones revealed reduced mortality and led to reduced or absent virus titers in the lungs. The Th cell response was antigen-specific and no CTL response could be detected. Only influenza viruses containing the relevant Th cell epitope were successfully cleared indicating that no bystander effect was involved. The transferred clonal Th cells did not, however, mediate protection directly but rather induced protective antibody responses
since transfer of Th cell clones into SCID mice did not mediate protection (Scherle et al., 1992). Furthermore, functional analysis of influenza virus-specific Th cell clones in vivo showed that Th cells specific for internal viral proteins provided 'cognate' help for B cell responses to HA, thus supporting intermolecular/intrastructural T-B interaction in the anti-influenza-specific antibody response (Scherle and Gerhard, 1986).

A further approach to study protective CD4+ T cell functions in influenza infection is the use of mice which are deficient in CD8+ CTLs (e.g. β2M-deficient mice or MHC class II-deficient mice). CD8-depleted mice or β2M-deficient mice eliminated influenza virus from the respiratory tract, thus indicating that influenza infection can be terminated by either CD4+ or CD8+ effector cells, but simultaneous removal of both subsets leads to a fatal outcome of the infection. However, the protective immune response may naturally be skewed to the development of CD8+ T cell-mediated effector functions (Eichelberger et al., 1991). On the other hand, it was demonstrated that β2M-deficient mice exhibited delayed virus clearance in the case of less virulent influenza strains but increased mortality after challenge with more virulent influenza strains (Bender et al., 1992).

Administration of anti-IFNγ antibodies to β2M-deficient mice caused a further delay in virus clearance but no switch to Th2-type Th cell responses, supporting again the idea that CD4+ T cells can control infection of certain strains of influenza (at least less virulent strains) either directly or more probably via induction of neutralizing antibody responses (Sarawar et al., 1994).

Similar findings were observed with Sendai virus infections. Analysis of the host response to Sendai virus infection in MHC class II-deficient mice revealed no impaired CD8+ CTLp generation (Hou et al., 1995). And similarly to influenza virus infections, delayed clearance of Sendai virus occurred in mice lacking MHC class I-restricted CD8+ T cells (Hou et al., 1992).

Th cells might not be strictly required for the induction of influenza-specific protective CTL responses (Tripp et al., 1995) but nevertheless, it has been shown that influenza-specific CD4+ T cell clones enhance the generation of influenza-specific secondary CTL responses in vitro: restimulation of purified CD8+ T cells required 'help' either by the presence of Th cells or by the addition of the corresponding Th cell supernatants whereby Th1 clones promoted significantly stronger memory CTL response inductions than Th2 clones. This could be correlated with IL-2 secretion being important for CD8+ T cell restimulation (Palladino et al., 1991).

Taken together, conditions could be defined in influenza and parainfluenza infections by modifying dose, route of infection and/or virulence of the virus strain in which either CD4+ (inducing antibody responses) or CD8+ T cell responses were sufficient
to induce protective immunity and to promote resolution from acute infection (Kast et al., 1986; Lightman et al., 1987; Mackenzie et al., 1989).

Poxviruses (ectromelia and vaccinia)

Host responses to poxvirus infection are varied and quite dependent on the host and virus species. For example, recovery from experimental ectromelia virus infection is strongly dependent on the generation of ectromelia-specific CTL responses. However, vaccinia in man or rhesus monkeys did not lead to classical primary CTL responses and thus cell-mediated cytotoxicity appears to be associated with ADCC and NK cells (Buller and Palumbo, 1991). Early appearing non-specific innate immune responses including IFNγ activation, inflammation and NK cell activation are beneficial but do not suffice to resolve infection. Specific immunity including antibody production, CTL activation and DTH responses are responsible for resolution of infection. Although antibody production does not appear to play a central role in controlling primary infection, it appears to be important in preventing reinfection (Buller and Palumbo, 1991)

Vaccinia infection in mice leads to the generation of primary CTL responses which are effective in viral clearance (Blanden, 1974; Hirsch et al., 1968). Nonetheless, in several situations CD4+ T cells are also able to confer protection against vaccinia infection (Kündig et al., 1993a; Oxenius et al., 1997b; Spriggs et al., 1992). A comparison of the anti-vaccinia protective capacity mediated by either CD4+ or CD8+ T cells revealed, that CD8+ effector T cells are more efficient in protection against vaccinia virus infection than CD4+ effector T cells (Binder and Kündig, 1991). Primed cytotoxic T cells which were capable to protect mice against intracerebral challenge with a 3-4 log scales more concentrated recombinant vaccinia virus inoculum as compared to primed CD4+ T cells. Another study using recombinant vaccinia viruses expressing either the VSV-derived G or N protein showed, that protection against recombinant vaccinia virus challenge of VSV-primed mice could be either mediated by CD4+ or CD8+ effector T cells - depending on the haplotype of the mouse strain and the recombinant protein and thus most probably depending on the availability of CD4+ or CD8+ T cell epitopes within the recombinantly expressed protein. However, if CD8+ T cell epitopes were available, they seemed to be dominant for protection (Kündig et al., 1993a). In line with this, β2M-deficient mice were shown to survive infection with high doses of vaccinia virus suggesting also a CD4+ T cell-mediated protective effector mechanism (Spriggs et al., 1992). In addition, MHC class II-restricted TCR transgenic mice with specificity for the LCMV GP were protected against infection with recombinant vaccinia virus expressing the LCMV GP but not against infection with recombinant vaccinia virus expressing an irrelevant protein (Oxenius et al., 1997b).
A direct role of cytokines such as IFNγ, TNFα and also IL-2 in recovery from vaccinia virus infection was demonstrated using recombinant vaccinia viruses expressing these cytokines at high levels at the site of infection. Immunodeficient mice recovered from infection with recombinant vaccinia virus expressing either IFNγ or IL-2 (Karupiah et al., 1990; Kohonen-Corish et al., 1990).

Another experimental approach providing evidence for a direct role of IFNγ in protection against vaccinia infection investigated the antiviral relevance of soluble mediators which may operate in the vicinity of virus-specific effector T cells. VSV-immune mice were challenged with a mixture of VSV-N and LCMV-GP expressing vaccinia viruses. Protection was observed against the VSV-N-expressing vaccinia virus in ovaries or testes after systemic challenge (but not against the coadministered LCMV-GP-expressing vaccinia virus). However, protection against both recombinant vaccinia viruses was observed in the brain after intracerebral challenge, indicating that 'bystander' protection by soluble mediators secreted by either CD4+ or CD8+ immune effector T cells is functional in organs where their diffusion is inhibited (e.g. in the brain) (Kündig et al., 1993).

Infection with ectromelia virus (a mouse pathogen which causes a generalized infection termed mousepox) leads to the induction of a vigorous CTL response, also in the absence of CD4+ T cells (Buller et al., 1987). Nonetheless, analysis of the different roles of CD4+ and CD8+ T cell subsets in the control of generalized ectromelia infection revealed that recovery from infection was strictly dependent on the CD8+ effector functions (Mullbacher et al., 1996) but CD4+ T cells were found to be required for the induction of optimal CTL responses. Thus, CD4-deficiency was associated with incomplete virus clearance and non-fatal persistence of ectromelia virus at low levels in most organs and at high levels in the skin (Karupiah et al., 1996). In addition, IFNγ has been attributed a central role in the recovery from mousepox infection: anti-IFNγ treatment (and to a much lesser degree anti-TNFα or anti-IFNβ treatment) resulted in virus persistence in all tissues tested except the primary site of infection, where virus clearance appears to be delayed. Anti-IFNγ treated mice finally succumbed to infection (Karupiah et al., 1993).

**Vesicular stomatitis virus (VSV)**

VSV belongs to the family of rhabdoviridae and is a relative of rabies (Wagner, 1987). VSV replicates only abortively in mice unless it reaches the central nervous system where it causes lethal paralysis (Wagner, 1987). VSV infection in mice induces a rapid Th-independent VSV-neutralizing IgM response which is followed by a largely Th-dependent VSV-neutralizing IgG response. VSV is not a polyclonal B cell activator but a very potent activator of VSV-specific B cells due to the efficient cross-linking capacity of highly repetitive organization of the VSV-G (representing
the determinant recognized by VSV-neutralizing antibodies) on the virion surface (Bachmann and Zinkernagel, 1996). The VSV neutralizing antibodies are mainly of the IgG2a isotype (Coutelier et al., 1987) and have been shown to effectively protect mice from lethal intravenous infection (Lefrancois, 1984). Thus, induction of VSV-specific Th cell responses plays a crucial role in protection against VSV infection via induction of Ig class-switched neutralizing antibodies. CD4-deficiency (e.g. MHC class II-deficient mice) or deficiency in CD40 or CD40L molecules impairs Ig class-switch and induction of B cell memory (Battegay et al., 1996; Oxenius et al., 1996); B cell deficiency results in a lethal outcome of VSV infection.

Most probably due to the efficient T-independent activation of VSV-specific B cells, only limited T help involved in cognate T-B interaction is required to induce Ig class switch. Transgenic mice expressing a MHC class II restricted TCR specific for a VSV-G epitope only show marginally enhanced kinetics of VSV-neutralizing antibody production upon infection with VSV (C. Burkhart and K. Maloy, personal communication). In addition, VSV-specific Th cell priming prior to VSV infection resulted in a secondary type antibody response of ELISA-binding VSV-specific antibodies but did not influence the kinetics of the protective VSV-neutralizing antibody response, suggesting that the 'bottle-neck' of the neutralizing antibody response is on the B cell side and not critically dependent on the kinetics of VSV-specific Th cell induction (Charan et al., 1986).

**Cytomegalovirus (CMV)**

CMV belongs to the family of the herpesviruses and infection is controlled by the immunocompetent host, however CMV infection of immuno-compromised hosts leads to a fatal outcome. For example, interstitial CMV pneumonia can cause mortality in irradiated, bone marrow transplanted leukemia patients and HIV-infected individuals often show manifestations of CMV disease as symptoms of AIDS (notably retinitis and colitis). CMV infection in immunocompetent hosts generally leads to viral latency in leukocytes (Jordan, 1978). In mice, CMV infections served as a model to investigate the contributions of different immune effector cell subsets in the resolution of acute infection. Adoptive cell transfer experiments demonstrated that CD8+ effector T cells are responsible for the control of acute MCMV infection whereas CD4+ T cells were dispensable for this short-term protection (Reddehase et al., 1988; Reddehase et al., 1987; Reddehase et al., 1985). Importantly, transfer of CD8+ T effector T cells prevented the lethal bone marrow aplasia which is caused by CMV-induced failure of hematopoietic stem cell generation (Mutter et al., 1988) and resulted in limited virus spread and tissue lesions (Reddehase et al., 1987). With respect to long-term virus control, CD4+ T cell depletion revealed delayed clearance of replicating virus in host tissues but nevertheless, protective CD8+ effector T cells
were generated which finally restricted virus replication to acinar glandular epithelial cells of the salivary glands (Jonjic et al., 1989). Thus, CD4+ T cells seem to influence the quality of CTL responses to MCMV which apparently has to be optimal for efficient clearance of MCMV from all host tissues. CMV infection of the lungs and subsequently developing interstitial pneumonia after intraperitoneal CMV infection was shown to be inhibited by adoptive transfer of CD4+ T cells, whereas this did not influence virus replication in the salivary glands, the preferential site of CMV infection in the mouse (Kadima-Nzuij and Craighead, 1990).

**Herpes simplex virus (HSV)**
Acute infection with HSV induces both cellular and humoral immune responses which are important in the resolution of acute disease but which do not prevent latent infection generating a life-long reservoir of HSV which periodically replicates in selected tissues (e.g. HSV-1 latently infects neurons and can remain in a quiescent state for years). Since antibodies cannot penetrate cells and since infected neurons do not express HSV-1 glycoproteins on their surface because they usually do not express MHC class I molecules and since neurons are usually not accessible by naive T cells, CD8+ T cells do not recognize and thus lyse HSV-harbouring neurons. Actually neither arm of the immune system is capable of protection from persistent infection. Control of acute HSV infection is generally mediated by CD8+ T cells (Sethi et al., 1983) but conditions could be identified where also CD4+ T cells play an important role for viral clearance (Nash et al., 1987). For example, mice deficient of CD4+ T cells showed a markedly increased susceptibility to herpes simplex virus infection of the skin and the nervous system leading to zosteriform lesions, whereas β2M-deficient mice were as resistant to challenge as were immunocompetent mice of the same genetic background (Manickan and Rouse, 1995). Vaccination with recombinant vaccinia viruses expressing herpes simplex virus immediate-early proteins induced protective immunity in BALB/c mice against herpes simplex virus challenge through CD4+ T cells of the Th1 phenotype without the involvement of CD8+ effector T cells (Manickan et al., 1995).

**Mammary tumor virus (MMTV)**
MMTV is a milk-transmitted type B retrovirus that encodes a superantigen in its 3' long terminal repeat. MMTV infection is correlated with a strong superantigen-induced Vβ-element restricted CD4+ T cell response. Different MMTV viruses have been described which exhibit superantigen activity for different Vβ elements on CD4+ T cells, thus 5-40% of the CD4+ T cell repertoire can possibly react with the encoded superantigen of a given MMTV strain (Acha-Orbea and MacDonald, 1995). The strong superantigen induced CD4+ T cell expansion can be measured within 3-4
days after infection and peaks 6 days after infection (Waanders et al., 1993; Webb et al., 1990). A few days after this initial expansion, unresponsiveness of the superantigen-reactive T cells is observed (Rammensee et al., 1989; Webb et al., 1990) and finally they disappear from the repertoire. This deletion is life-long and occurs in the presence or absence of the thymus (Acha-Orbea and MacDonald, 1995). Parallel to the expansion of superantigen-reactive CD4+ T cells a strong polyclonal B cells activation leads to cell proliferation and differentiation into Ig-secreting antibody forming cells (Held et al., 1993a). An antiviral antibody response can be detected (predominantly of the IgG2a and IgG2b isotypes) but these MMTV-specific antibodies do not seem to drastically interfere with established infection (Acha-Orbea and MacDonald, 1995). It has been repeatedly suggested that B cells are preferentially infected by MMTV with concomitant integration of the viral DNA into the B cell genome and thus are the prominent APCs during superantigen-induced T cell activation. This has also been been proposed to be a cause for the observed superantigen-reactive CD4+ T cell unresponsiveness (Eynon and Parker, 1992; Fuchs and Matzinger, 1992). However, the tremendous activation of superantigen-reactive CD4+ T cells leads to 'cognate' help for MMTV infected B cells which finally induces the observed polyclonal B cell activation and expansion (Held et al., 1993b). This polyclonal B cell activation is clearly dependent on the presence and activation of superantigen-reactive CD4+ T cells and vice versa lack of productive infection in the absence of superantigen-reactive CD4+ T cells was observed (Held et al., 1993b). Although MMTV seems to be a T-independent B cell activator, MMTV infected B cells have a short survival time in the absence of additional factors or functions provided by superantigen-reactive CD4+ T cells (Acha-Orbea and MacDonald, 1995). After stable MMTV infection has been achieved, infection ultimatively spreads to the mammary gland and leads to virus transmission via the milk.

Thus, in contrast to many viruses which aim to evade efficient immune responses for their survival, MMTV deliberately uses the host immune response for its own purposes. In particular, the superantigen-induced activation and deletion of Vβx-expressing CD4+ T cells and the expansion of infected B cells is an instrumental step for successful persistent infection and stable integration of the viral genome. A different strategy of using the host immune response for its own purposes is pursued by HIV which uses CD4+ T cells and macrophages as targets of infection thereby slowly but progressively interfering with host immune responses.
Conclusions

The coevolution of pathogens and the immune system in their respective hosts has established a subtle balance between the parasitic usurpation of the host cellular functions by the virus with the aim to survive and most importantly to be transmitted whereas the defence mechanisms of the host aim at resolving infection without causing fatal immunopathology. Both sides have developed multiple mechanisms to achieve their respective goals which have become increasingly apparent over recent years.

Focussing on the role of virus-specific CD4+ effector T cells in viral infections, it might not be surprising that generalizations are almost impossible to make. Depending on the characteristics of a virus (cytopathogenicity, cell tropism, virulence, persistence etc.) CD4+ T cells may play a more or less crucial role in the resolution of infection. Generally, acute infections with non-cytopathic viruses such as LCMV or HBV are resolved by cytotoxic CD8+ effector T cells whereas infections with cytopathic viruses may be either only controlled by virus-neutralizing antibodies (e.g. VSV) or by variable contributions of CD4+ T cells, antibodies and CD8+ T cells (e.g. influenza, vaccinia virus). In the latter cases, the virus dose, the route of infection and the virulence of the virus strain determines - at least partly, whether or not CD4+ T cells (and/or antibodies) can be sufficient for viral clearance. The diverse effector mechanisms of Th cells (secretion of cytokines, cognate help for B cells, help for CTL induction, DTH reaction) are of different importance in different viral infections. Viruses which are sensitive to antivirally active cytokines (e.g. vaccinia) may be entirely controlled by T cell-dependent cytokine secretion. Cognate help for antiviral antibody production is not only important for the resolution of primary infections of some cytopathic viruses and especially of those viruses hidden in immuno-privileged sites, but also for the induction of memory antibody titers which represent the most potent protection against reinfection.

Usually the generation of cytotoxic T cell responses upon acute infection is not - or only partly - dependent on CD4+ T cell functions. However, the situation may be different for the long-term control of infections with viruses which have the tendency to persist - even at levels below the threshold of detection by normal assays. Optimal CD4+ T cell-dependent induction of CTL activity, sustainment of prolonged CTL activity, optimal memory CTL function and/or the induction of (neutralizing) antibody responses might be crucial for the long-term protection against many virus infections.

Viral infections generally induce Th cells exhibiting a Th1 phenotype which contribute to the induction of cellular immune responses that are of crucial importance in the defence against intracellular pathogens. The mechanism(s)
governing this skewing of the Th cell phenotype is still unclear but it may depend on
the nature of viruses to induce innate immune responses dominated by IFNγ secretion, NK cell and macrophage activation.

The APC requirements for Th cell induction upon viral infections are not as clearly
defined as for soluble antigens but may depend on the viral host cell tropism, replication kinetics and the cytopathic or non-cytopathic nature of the virus. Some viruses exhibit additional pathways for antigen presentation on MHC class II molecules whereby intracellularly synthesized viral antigens are able to directly load MHC class II molecules.

Taken together, although virus-specific CD4+ T cells have been shown to promote by themselves recovery from virus infections in some selected infectious systems, virus-specific cytotoxic CD8+ effector T cells are apparently dominant in the resolution of acute infections by non-cytopathic viruses and important for the resolution of several infections by cytopathic viruses. However, in the latter case, neutralizing antibody responses or T cell-secreted cytokines may additionally be of crucial relevance for clearance of the virus. In general, the cooperations of the different arms of the immune system, the CD4+ T cells representing one central regulator of these arms particularly important for generation of protective anti-viral antibody responses, offer the optimal defence against viral infections and such optimal cooperation seems to become particularly important in persisting viral infections.
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Appendix: Abstracts

**TAP1-independent loading of class I molecules by exogenous viral proteins**

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Presentation of peptides derived from endogenous proteins on class I molecules needs functional TAP peptide transporters. To reveal whether class I-associated presentation of exogenous proteins also required the presence of TAP transporters, we assessed *in vitro* the ability of spleen cells and macrophages from TAP1-deficient mice (TAP1−/−) to present peptides from exogenous recombinant viral proteins on their class I molecules. We found that recombinant glyco- and nucleoprotein from lymphocytic choriomeningitis virus and nucleoprotein from vesicular stomatitis virus were presented as efficiently by TAP1−/− cells as by control cells. Peptide regurgitation was not involved. Since particulate, non-replicating antigens can efficiently prime anti-viral cytotoxic T cells *in vivo*, this new, TAP-independent pathway of class I-associated antigen presentation may be applicable for vaccine strategies.

T cell development in CD8⁻/⁻ mice: thymic positive selection is biased toward the helper phenotype

Martin F. Bachmann, Annette Oxenius, Tak W. Mak and Rolf M. Zinkernagel

The CD4 and CD8 molecules are involved in T cell differentiation and activation. Nevertheless, thymic maturation of helper T cells has been shown in the absence of the CD4 molecule. These CD4-deficient helper T cells expressed αβ-TCR and were able to control *leishmania* infections and to mediate Ab class switch. Using mice deficient for the CD8 α-chain, we investigated whether a similar cytotoxic T cell population was generated in the absence of the CD8 coreceptor. A CD8-deficient cytotoxic T cell population corresponding to the described CD4-deficient helper T cell population was virtually absent both functionally and physically. These results support the idea that thymic maturation is asymmetrical and strongly biased toward the helper phenotype.

Similar ligand densities required for restimulation and effector function of cytotoxic T cells

Annette Oxenius and Martin F. Bachmann

This study compared ligand densities on antigen presenting cells (APC) needed for restimulation of in vivo primed T cells and for T cell effector function. Spleen cells of lymphocytic choriomeningitis virus (LCMV) primed mice were restimulated in vitro with graded amounts of virus derived peptides using macrophages or a cloned dendritic cell line as APC. To test for effector function of these cytotoxic T cells, the same APCs pulsed with graded amounts of the peptides were used as target cells in an in vitro 51-Cr release assay. The same peptide concentration that rendered an APC restimulatory for primed CTLs also rendered it susceptible for lysis by the same CTLs.

Thus, restimulation of in vivo primed T cells - measured either by proliferation or cytotoxic effector function - or sensibilization of target cells for lysis require similar ligand densities and are therefore, contrary to expectations, governed by similar overall avidity-thresholds. These results have implications for CTL memory.

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Abbreviations

APC: antigen presenting cell
β2M: beta-2-microglobulin
BFA: brefeldin A
CLIP: class II associated invariant chain peptide
CTL: cytotoxic T lymphocyte
CTLp: cytotoxic T lymphocyte precursor
DC: dendritic cell
DNA: desoxyribonucleic acid
DTH: delayed type hypersensitivity
EBV: Ebstein-Barr virus
ER: endoplasmic reticulum
FDC: follicular dendritic cell
G, GP: glycoprotein
GM-CSF: granulocyte macrophage colony stimulating factor
IFN: interferon
Ig: immunoglobulin
Ii: invariant chain
IL: interleukin
HEL: hen egg lysozyme
LCMV: lymphocytic choriomeningitis virus
MHC: major histocompatibility complex
N, NP: nucleoprotein
NK cell: natural killer cell
OVA: ovalbumin
RNA: ribonucleic acid
TCR: T cell receptor
Th cell: T helper cell
TNF: tumor necrosis factor
VSV: vesicular stomatitis virus
Curriculum vitae

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List of Publications

First author publications:


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