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Priming and Tolerance of Lymphocytic Choriomeningitis Virus Specific Cytotoxic T Cells

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Content

Summary

Infection of mice with lymphocytic choriomeningitis virus (LCMV) elicits a vigorous cytotoxic T cell response that is instrumental to virus control. The three studies that form this thesis use the LCMV murine infection model to address fundamental questions concerning the induction and tolerization of cytotoxic T cell responses.

The first chapter of this thesis is formed by two studies investigating the immunodominance of the CTL response against LCMV in C57BL/6 mice. In the first study we test the hypothesis that competition for access to antigen presenting cells by T cells of different specificities influences the immunodominance of CTL responses. This hypothesis was proposed in a recent study using ovalbumin as a model antigen, and it received considerable attention. Using LCMV-specific TCR transgenic CD8+ T cells we analyzed whether an increased frequency of CTL precursors specific for one LCMV epitope would inhibit the priming of CTL specific for other LCMV epitopes. Adoptive transfer of TCR transgenic T cells inhibited the endogenous response of the same specificity in a dose dependent manner. However, even when transfer of more than 10^6 TCR transgenic cells was combined with weak priming, no reduction of responses to other epitopes was observed. Thus, competition for antigen presenting cells by CTLs of different specificities is not of functional relevance in antiviral immune responses.

The second study investigates the dependence of immunodominance on viral load. The CTL response against LCMV is directed against both structural proteins, the nucleoprotein (NP) and the glycoprotein (GP). We found that a small load of LCMV led to immunodominance of NP-CTL, whereas a large viral load resulted in dominance of GP-CTL. We demonstrate that an infected cell presents NP-derived peptides considerably earlier than GP-derived peptides. This results in a stronger stimulus for NP-specific CTL as compared to GP-specific CTL, which led to preferential priming of NP-CTL at low viral loads but also made NP-CTL more susceptible to exhaustion at higher viral

loads. This is the first study describing that immunodominance is not fixed after infection with a given pathogen, but varies with the viral load instead.

The study that forms the second chapter of this thesis investigates the role of dendritic cells (DC) in the maintenance of self tolerance of cytotoxic T cells. DC are known as inducers of immune responses par excellence. They also appear to be responsible for the induction of peripheral T cell tolerance under steady state conditions. To investigate these controversial functions without adoptive transfers, we generated a system that allows inducible antigen presentation by DC *in vivo*. We found that presentation of LCMV-derived CTL epitopes by resting DC resulted in antigen-specific tolerance, which could not be broken by subsequent infection with LCMV. On the other hand, antigen presentation by activated DC primed endogenous CTL to expand and to develop protective effector function.

Zusammenfassung

Die Infektion von Mäusen mit dem Lymphozytären Choriomeningitis Virus (LCMV) löst eine heftige zytotoxische T Zell (CTL) Antwort aus, die entscheidend für die Kontrolle des Virus ist. Die drei Arbeiten, aus denen sich diese Dissertation zusammensetzt, untersuchen anhand dieses Mausmodells grundlegende Fragen zur Induktion und Tolerisierung zytotoxischer T Zell Antworten.

Das erste Kapitel dieser Dissertation besteht aus zwei Arbeiten zur Immundominanz der CTL Antwort gegen LCMV in C57BL/6 Mäusen. In der ersten Arbeit überprüfen wir die Hypothese, dass Konkurrenz von T Zellen unterschiedlicher Spezifität um antigenpräsentierende Zellen die Immundominanz von CTL Antworten beeinflusst. Diese Hypothese wurde in einer kürzlich veröffentlichten Arbeit, in der Ovalbumin als Modellantigen verwendet wurde, vorgeschlagen und erhielt erhebliche Beachtung. Unter Verwendung von LCMV spezifischen T Zell Rezeptor (TCR) transgenen T Zellen haben wir untersucht, ob eine erhöhte Frequenz von CTL Vorläufern mit Spezifität für ein bestimmtes LCMV Epitop das Priming von CTL mit Spezifität für andere LCMV Epitope hemmen würde. Durch adoptiven Transfer von TCR transgenen T Zellen wurde die endogene Antwort der gleichen Spezifität dosisabhängig gehemmt. Eine Beeinträchtigung der Antwort gegen andere Epitope wurde hingegen selbst bei Transfer von mehr als 10^6 TCR transgenen T Zellen, kombiniert mit schwachem Priming, nicht beobachtet. Konkurrenz von T Zellen verschiedener Spezifität um antigenpräsentierende Zellen hat daher keine funktionelle Bedeutung für antivirale Immunantworten.

Die zweite Arbeit beleuchtet die Anhängigkeit der Immundominanz von der Viruslast. Die CTL Antwort gegen LCMV richtet sich gegen beide strukturelle Proteine des Virus, das Nukleoprotein (NP) und das Glykoprotein (GP). Wir beobachteten, dass eine geringe Viruslast zu Immundominanz von NP-CTL führte, während eine hohe Viruslast zu Dominanz von GP-CTL führte. Wir konnten zeigen, dass eine infizierte Zelle Peptide aus dem Nukleprotein

deutlich früher präsentiert als Peptide aus dem Glykoprotein. Dadurch werden NP spezifische CTL verglichen mit GP spezifischen CTL stärker stimuliert. Dies führt zu bevorzugtem Priming von NP-CTL bei tiefer Viruslast macht NP-CTL aber auch empfindlicher für Exhaustion bei höher Viruslast. Diese Arbeit zeigt daher zum ersten Mal, dass die Immundominanz in der Infektion mit einem bestimmten Virus nicht festgelegt ist sondern von der Viruslast abhängt .

Die Arbeit, die das zweite Kapitel dieser Dissertation bildet, behandelt die Rolle von dendritischen Zellen (DC) für die Aufrechterhaltung der Selbst-Toleranz von zytotoxischen T Zellen. DC sind bekannt als exzellente Stimulatoren von T Zell Antworten. Sei scheinen zudem für das Etablieren der peripheren T Zell Toleranz unter homöostatischen Bedingungen verantwortlich zu sein. Wir haben ein System entwickelt, das uns erlaubt, diese gegensätzlichen Funktionen unter Vermeidung von adoptiven Zelltransfers zu untersuchen. Wir haben beobachtet, dass die Präsentation von CTL Epitopen aus LCMV durch ruhende DC zu antigenspezifischer Toleranz führte, die nicht durch eine darauffolgende Infektion mit LCMV gebrochen werden konnte. Antigenpräsentation durch aktivierte DC löste hingegen eine Effektor CTL Antwort aus, welche die Maus vor einer nachfolgenden LCMV Infektion schützte.

Aim of the study

Cytotoxic T lymphocytes (CTL) are important effector cells that help to protect higher vertebrates from infections with viruses and other pathogens that replicate inside the host cells and are therefore out of reach of the soluble effector molecules of the immune system, such as antibodies and complement proteins. When a cytotoxic T cell detects an infected cell it can kill this cell by the release of granules containing cytotoxic molecules, thereby limiting pathogen spread. Like a detective that browses the dustbin of a house to get clues on its residents, a CTL inspects the degradation products of the proteins synthesized within a cell for the presence of pathogen-derived material. The degradation products are presented to the cytotoxic T cells as small peptides of 8-10 amino acids that are bound to MHC class I molecules expressed on the cell. As loading of the peptide onto MHC class I takes place in the endoplasmic reticulum of the cell, the so-called MHC restriction of antigen recognition by CTL ensures that the peptide that is recognized has indeed been produced inside the cell.

Of the many peptides that can be generated by the degradation of the various proteins of a pathogen, only a few meet the structural requirements to be efficiently presented on MHC class I and among those few, the cytotoxic T cell response is focused mainly on one or several so called immunodominant determinants. Immunodominance is seen in all CTL responses to pathogens. A detailed knowledge of factors that determine which specificities will dominate the CTL response against a pathogen is important for the understanding of mechanisms underlying the induction of CTL responses, the evasion of pathogens from CTL responses, as well as for the design of vaccines. Several factors that shape immunodominance of CTL responses to pathogens have been identified. These either concern the antigen presenting cell, such as the efficiency of the processing and presentation of the antigenic peptides, or the responding T cells, such as the frequency of specific precursors for a given peptide epitope. In the two studies that form the first part of this thesis we have investigated factors that influence the

immunodominance of the CTL response against a viral infection using the well-characterized murine model of infection with the lymphocytic choriomeningitis virus (LCMV). The first study addresses the question whether and how the frequency of naïve T cells specific for one determinant can influence the response against other determinants. The set of experiments described in the second study analyzes the influence of the virulence of different virus isolates and of the size of the inoculum on the immunodominance of the CTL response against LCMV.

With their ability to kill virtually all types of cells, cytotoxic T cells can cause major tissue destruction and represent a potential danger to the integrity of the organism. As the majority of peptides that are presented in complex with MHC-class I molecules of a cell are not derived from pathogen encoded proteins but from the degradation of cellular proteins, the cytotoxic T cells have to be instructed about which peptides indicate the presence of harmful pathogens and therefore target the cell for destruction. Elimination of autoreactive T cells during their development is the main mechanism to prevent CTL activity towards cells that are not infected. However, it has become clear that this instruction process is unable to delete all potentially autoreactive T cells and additional mechanisms must be at work to maintain this so called “self tolerance”. It could be shown that, although activated cytotoxic T cells can detect infection in and react against almost all types of cells, the ability to activate CTL is restricted to a specialized class of cells, the professional antigen presenting cells (APC). Dendritic cells are thought to be the APC that are central to the induction of CTL responses. To be able to activate naïve T cells, dendritic cells have to undergo a process of differentiation that is referred to as maturation. This process is triggered by components of the evolutionary old innate defence system that recognize molecules that are characteristic for pathogens such as bacterial cell wall components or viral nucleic acids. Mature dendritic cells can be generated in vitro. If these mature DC are loaded with antigenic peptide and are injected into mice, they readily activate naïve T cells. The outcome of antigen presentation by immature DC has been difficult to study as isolation or even *in*

vitro culture of DC already induces maturation of the cells. To overcome this limitation we have developed a novel transgenic mouse model that which allows us to induce the presentation of antigenic peptides on dendritic cells directly *in vivo*. In the study that forms the second part of this thesis we describe both the generation of this mouse model and experiments designed to investigate how the activation status of the DC can determine the outcome of antigen presentation.

Results Part I: Immunodominance of the CTL Response Against LCMV

Competition for antigen presenting cells by CTLs of different specificities is not functionally important during induction of antiviral responses

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Abstract

The hypothesis that T cell competition for access to APC influences priming of CTL responses is a controversial issue. A recent study using OVA as a model antigen supports this hypothesis and received considerable attention. However, using a comparable approach, we reached a different conclusion. We analyzed whether TCR transgenic T cells specific for lymphocytic choriomeningitis virus (Zinkernagel and Doherty, 1979)GP33-41/D^b could inhibit the priming of endogenous responses against GP33-41 and against two other, lymphocytic choriomeningitis virus glycoprotein-derived CTL epitopes. After priming with different stimuli, GP33-41/D^b-specific TCR transgenic T cells reduced the endogenous GP33-41/D^b response in a dose-dependent way, but all other endogenous responses were unaffected. Even when $>10^6$ TCR transgenic cells were combined with weak priming, no reduction of responses other than of those specific for GP33-41/D^b was observed.

Thus, competition for antigen presenting cells by CTLs of different specificities is not of functional relevance in antiviral immune responses.

Introduction

In the control of infections with viruses or intracellular bacteria, $CD8^+$ T cells have been shown to be of crucial importance in the control of infections (Zinkernagel and Doherty, 1979). $CD8^+$ T cells recognize 8-10 aa long peptide fragments presented on the cell surface by major histocompatibility complex (MHC) class I molecules (Rammensee et al., 1993).Only a few of the numerous peptides encoded by pathogens are able to activate specific $CD8^+$ T cells, and responses to these epitopes are often dominated by one or two epitopes. This phenomenon is termed immunodominance (Chen et al., 2000; Sercarz et al., 1993; Yewdell and Bennink, 1999). Knowledge of mechanisms that influence immunodominance is important to the understanding of $CD8^+$ T cell priming, viral evasion mechanisms or the design of vaccines. Several factors that contribute to immunodominance have been identified, and their relative importance seems to depend on the experimental system used.

These include the efficiency of epitope generation by the antigen processing machinery, the affinity of the peptide for the presenting MHC Class I molecule, and the abundance of T cells responding to this particular peptide-MHC Class I complex (Chen et al., 2000; Chen et al., 1994; Deng et al., 1997; Eisenlohr et al., 1992; Ossendorp et al., 1996; Restifo et al., 1995; Sette et al., 1994; Yewdell and Bennink, 1999). Antigenic competition, in which immune responses to one determinant are inhibited by simultaneous exposure to (other) antigens on the same APC (immunodomination), has recently been suggested to contribute to immunodominance (Chen et al., 2000; Grufman et al., 1999; Kedl et al., 2000; Wolpert et al., 1998). The observation that, upon secondary challenge (Busch and Pamer, 1999; Rees et al., 1999; Savage et al., 1999) or in the memory phase (Slifka and Whitton, 2001) of an immune response, the affinity of the responding T cells increases without evidence for affinity maturation of the TCR is also interpreted in terms of T cell competition. Thus, T cells with a higher affinity must have an advantage in secondary responses (where usually the antigenic load is low), suggesting that T cells compete for antigen. Obviously, in the aforementioned experiments (Busch and Pamer, 1999; Rees et al., 1999; Savage et al., 1999; Slifka and Whitton, 2001) competition is studied between T cells with the same specificities but different TCR affinities.

Whether T cells with different specificities compete with each other for access to APC has been addressed in three systems with studiesstudy using: 1) C57BL/6-anti-BALB.B minor histocompatibility antigens (Grufman et al., 1999; Wolpert et al., 1998); 2) H-Y and $B6^{dom1}$ minor histocompatibility antigens (Loyer et al., 1999; Pion et al., 1999); and 3) OT-1 transgenic T cells (specific for the OVA-derived peptide SIINFEKL(S8L)/K^b) combined with infection with recombinant vaccinia virus (VV) expressing OVA or priming by dendritic cells (DC) loaded with antigenic peptides (Kedl et al., 2000). In the studies using transplantation antigens, elimination of APC by dominant CTL (faster, more) was suggested as a mechanism, whereas the study using OVA claimed that T cell competition for access to APC was responsible.

To study whether T cell competition for antigen-bearing APC is a mechanism of general importance in immunodominance, we adoptively transferred titrated numbers of TCR transgenic $CD8^+$ cells (318, specific for lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived 33-41/D^b) together with priming for LCMV-derived CTL epitopes. LCMV is a potent system for studying immunodominance, as a strong and well-characterized αCTL response directed against three LCMV glycoprotein-derived immunodominant epitopes (GP33-41/D^b, GP34-41/K^b, GP276-286/D^b) is elicited. To prime different numbers of specific CTL, mice were injected with LCMV, GP1-60 transgenic DC, or VV-G2 (expressing LCMV glycoprotein). Analysis of the endogenous CTL reponse against all immunodominant epitopes showed that no inhibition of endogenous CTL response against epitopes other than GP33 occurred.

Materials and Methods

Mice

C57BL/6 mice were obtained from the Institut für Labortierkunde (University of Zürich, Zürich, Switzerland). B6.PL (Thy1.1) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Transgenic mice expressing the LCMV glycoprotein aa 1-60 (H8 mice) have been described previously (Ehl et al., 1998), as have the mice expressing the P14 TCR recognizing LCMV GP33-41/H-2D^b (318 mice (Pircher et al., 1990)). For adoptive transfer experiments, 318 x B6.PL (318.PL) mice were used as donors. All mice were on a C57BL/6 background. Male mice of 6-10 weeks of age were used.

Viruses

LCMV-WE was originally obtained from Dr. F. Lehmann-Grube (University of Hamburg, Hamburg, Germany) (Lehmann-Grube, 1971) and was propagated on L929 cells at a low multiplicity of infection. Recombinant Vaccinia Virus (rVV) encoding the LCMV glycoprotein (VV-G2) was obtained from Dr. D. Bishop (Institute Of Virology, Oxford, UK) and was propagated on BSC40 cells (Hany et al., 1989).

Generation of bone marrow-derived dendritic cells

Bone marrow-derived DC were generated from femora of H8 mice as previously described (Ludewig et al., 1998). DC were cultured in the presence of the agonistic anti-CD40 monoclonal antibody FGK45 (50mg/ml, (Schoenberger et al., 1998)) during the last 48 h.

Adoptive transfer and infection

318.PL $CD8^+$ splenocytes were positively selected on a VS/LS column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The selected population contained >95% $CD8^+Thy1.1^+$ cells. $CD8^+$ cells were injected into C57BL/6 mice. After $CD8^+$ transfer, mice were infected with 10^2 plaque forming units (pfu) LCMV-WE or with 10^6 pfu VV-G2. Alternatively, mice were injected immediately after adoptive transfer with H8 DC. Virus, $CD8^+$ cells and DC were injected in 0.2 ml balanced salt solution into the tail vein.

Antibodies, tetramers and flow cytometry

MHC Class I tetramers were produced as previously described (Gallimore et al., 1998b). Phycoerythrin (PE)-labeled anti-CD90.1 (Thy1.1) (clone HIS51) and Fluorescein isothiocyanate (FITC)-labeled anti-CD8a (clone 53-6.7) were obtained from PharMingen (San Diego, CA).

At indicated timepoints, blood samples were stained for 10 minutes at 37°C with tetramer, followed by staining with anti-CD8FITC + anti-Thy1.1PE for 30 minutes at 4°C. Samples were washed twice, erythrocytes were lysed with FACS Lysis Solution (BD Biosciences, Mountain View, CA), and analysed on a FACScan using Cell Quest software (BD Biosciences).

Determining viral titers

LCMV titers were measured in the spleens of infected mice at the indicated timepoints. Organs were homogenized and monolayers of MC57G cells were infected with 10-fold dilutions of the homogenate for 48 h. LCMV was detected by intracellular staining with a monoclonal rat anti-LCMV nucleoprotein antibody (VL4) as described (Battegay et al., 1991b). VV titers were determined in ovaries: confluent monolayers of BSC40 cells were

Infected with 10-fold dilutions of the ovary homogenates. Plaques were visualised by crystal violet after 48 h.

Results

Transferred LCMV GP33-41/D^b-specific TCR transgenic (318) $CD8^+$ cells inhibit the endogenous GP33-41/D^b response, but leave other endogenous, LCMV-specific responses intact.

Infection of C57BL/6 mice with 100 pfu LCMV-WE primed substantial numbers of $CD8^+$ cells for each of the three LCMV GP-derived immunodominant epitopes. Mice were bled 12 days after infection (peak of the response in the blood) and cells were stained with Cychrome-labeled tetramers, PE-labeled anti-Thy1.1 and FITC-labeled anti-CD8 antibodies. The percentage of endogenous specific CTL was determined within the $CD8^+Thy1.1^-$ population. We found that 14.9% of $CD8^+$ cells were specific for GP33, 10.4% for GP34, and 3.0% for GP276 (Figure 1). Adoptive transfer of titrated numbers of naïve, MACS-purified $CD8^+$ cells from 318.PL mice that carry a transgenic TCR specific for LCMV GP33 in ~50% of their $CD8^+$ cells together with infection with 100 pfu LCMV-WE reduced priming of the endogenous (Thy1.1-) $CD8^+$ cells for GP33 in a dose-dependent way (Figure 1), demonstrating that competition between T cells of the same specificity occurred. The total number of GP33 CTL (endogenous + transferred) in the spleen 8 d p.i. varied between 3.4-8.9 x 10^6 GP33 CTL, and was relatively independent of the number of adoptively transferred 318 cells (not shown). This shows that increasing the number of CTL precursors does not necessarily increase the response (Laouar and Crispe, 2000; Smith et al., 2000). The competition observed by us is well in line with the experiments published by Kedl, et al. (Kedl et al., 2000), who found that adoptive transfer of TCR transgenic, SIINFEKL/K^b-specific OT-1 cells inhibited the priming of SIINFEKL/K^b-specific $CD8^+$ cells by VV-OVA. However, the endogenous response to the other two LCMV GP-derived immunodominant epitopes was not affected by adoptive transfer of 318.PL cells: even after transfer of as

many as 10^6 TCR transgenic $CD8^+$ cells, there was no difference to nontransferred LCMV-WE infected mice with respect to the percentage of $CD8^+Thy1.1^-$ cells specific for GP34 (9.1%) or for GP276 (2.7%), whereas the endogenous GP33 response was drastically reduced to 0.6% (Figure 1). We found similar results at d 8 and d 30, and also when we compared blood and spleen (data not shown). In addition, similar results were obtained after infection of mice with 10^6 pfu LCMV-WE (data not shown). Transfer of 318 cells reduced LCMV loads from days 4-5 on; titers in the spleen were similar in mice that received no cells or $3x10^4$ or 10^6 TCR transgenic cells on days 2 and 3 p.i. On day 4 p.i. $3x10^4$ 318 cells did not reduce titers, but 10^6 318 cells reduced titers twofold. On day 5 p.i. $3x10^4$ 318 cells reduced titers twofold, and 10^6 318 cells reduced titers 100-fold.

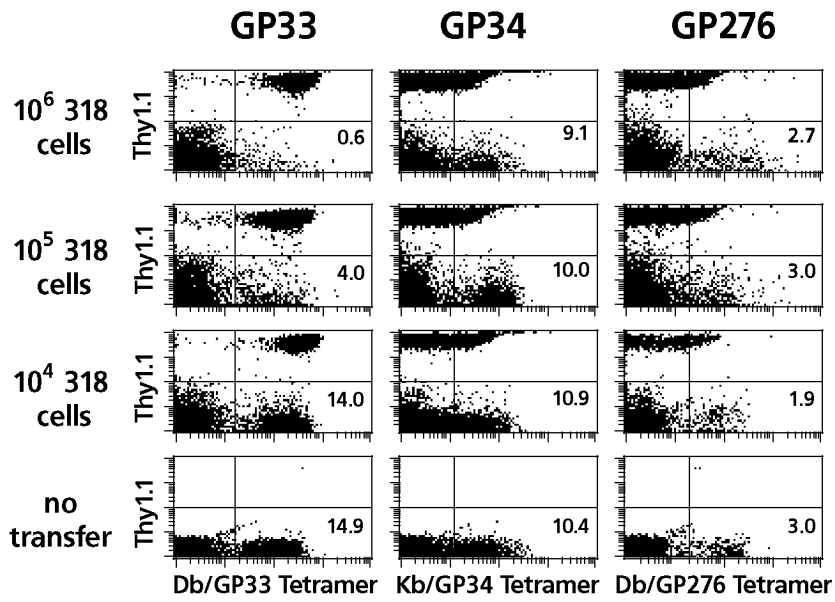

Figure 1: **Transferred LCMV GP33-41/Db-specific TCR transgenic (318) CD8+ cells compete against the endogenous GP33-41/Db response, but not against other endogenous, LCMV-specific responses**.

C57BL/6 mice were adoptively transferred with titrated numbers of 318.PL CD8+ cells and were infected with 100 pfu LCMV-WE. 12 d after infection, PBL were stained with Cychrome-labeled GP33-41Db-, GP34-41Kb-, or GP276-286Db-tetramers, with anti-CD8-FITC and with anti-Thy1.1-PE as described in Materials and Methods. Data were analyzed by gating on CD8+ cells, Thy1.1+ cells represent the adoptively transferred 318 cells, whereas Thy1.1- cells represent the endogenous response. The number given in the lower right quadrant represents the % tetramer+ cells of theendogenous response. Representative stainings are shown.

Adoptively transferred LCMV GP33-41/D^b-specific TCR transgenic $CD8^+$ cells do not inhibit priming of endogenous $CD8^+$ responses to other LCMV-derived epitopes, not even when priming is weak

In the experiments described above, we found no evidence for T cells that inhibit priming of T cells specific for other epitopes presented by the same APC. Because infection with LCMV, a noncytopathic virus that is known to replicate well in immunocompetent mice, primes a vehement CTL response, we reasoned that priming with a weaker infectious agent that putatively gives rise to less LCMV-presenting APC might reveal evidence for T cell competition for access to antigen-bearing APC. Therefore, we transferred 318 $CD8^+$ cells into C57BL/6 mice infected with 100 pfu LCMV-WE or with 10^6 pfu VV-G2, and measured the endogenous CTL reponse against all immunodominant LCMV GP-derived CTL epitopes expressed by both pathogens 9 d later (peak response in the blood after VV priming). Infection with LCMV-WE confirmed the data shown in Figure 1: As little as 10^4 318 $CD8^+$ cells (equivalent to 5000 GP33-specific cells) already inhibited the priming of endogenous GP33-specific response to some extent; considerable (LCMV-WE) to almost complete (VV-G2) inhibition was observed after transfer of 10^5 318 $CD8^+$ cells. No inhibitory effect was seen on the priming of the two other immunodominant specificities, not even after transfer of 10^6 318 $CD8^+$ cells (Figure 2A). Even after very weak priming with VV-G2 (compare the percentages of tetramer positive cells of Figure 2B with those of Figures 2A), we found no evidence for inhibition of priming of the endogenous GP34 or GP276 response, whereas priming of the endogenous GP33 response was clearly inhibited (Figure 2B). We found no evidence for enhanced VV elimination due to transferred 318 cells (up to10^6) as measured in the ovaries 5 d after infection. Importantly, tetramer positive cells of all three specificities were fully functional independent of the number of transferred 318.PL $CD8^+$ cells as determined by chromium-release assay (not shown).

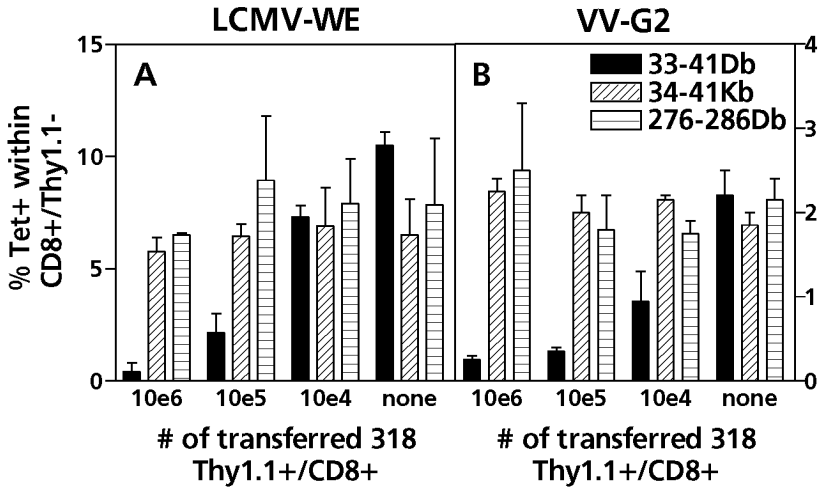

Figure 2 : Transferred LCMV GP33-41/D^b-specific TCR transgenic (318) CD8$^+$ cells compete against the endogenous GP33-41/D^b response, but not against other endogenous, LCMV-specific responses, and this is independent of antigenic load or the degree of priming.

Titrated numbers of 318.PL CD8$^+$ cells were adoptively transferred into C57BL/6 mice, followed by infection with (A) 100 pfu LCMV-WE, (B) 10^6 pfu VV-G2 (expressing LCMV GP). FACS staining was done as described in Figure 1. Data represent the mean +/- SD of 3 individual mice bled at day 9 after infection. This experiment was performed three times with similar results.

Transferred LCMV GP33-41/D^b-specific TCR transgenic (318) CD8$^+$ cells compete against the endogenous GP33-41/D^b response, but not against the GP34-41/K^b response after priming with GP1-60 expressing DC (H8 DC)

We primed C57BL/6 mice with titrated numbers of transgenic H8 DC (Ehl et al., 1998) combined with transfer of titrated numbers of 318.PL CD8$^+$ cells. H8 DC continuously present both GP33 and GP34 and have the advantage over peptide-loaded DC that off-rates of peptides are not confusing the experimental system. Mice were bled at d 7, at which the peak response after DC priming is seen. We found that mice injected with 10^5 H8 DC together with titrated numbers of TCR transgenic CD8$^+$ cells displayed a dose-dependent inhibition of priming of endogenous GP33 CTL, but unaffected priming of GP34 CTL (Figure 3). Similar results were obtained if mice were primed with 10^6 H8 DC (not shown).

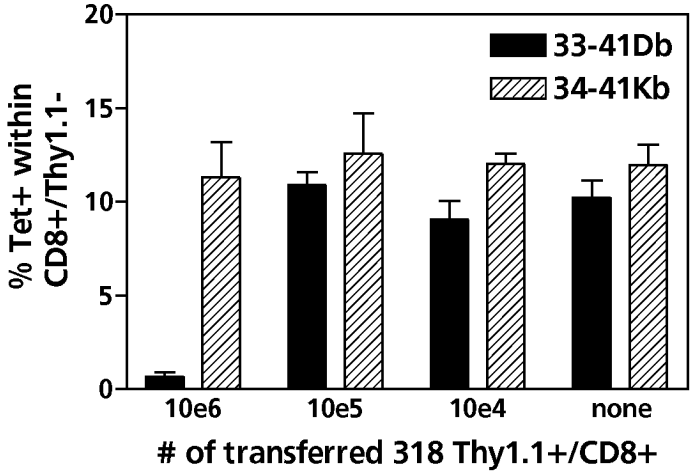

Figure 3 : Transferred LCMV GP33-41/D^b-specific TCR transgenic (318) CD8$^+$ cells compete against the endogenous GP33-41/D^b response, but not against the GP34-41/K^b response after priming with 10^5 GP1-60 expressing DC (H8 DC).

Titrated numbers of 318.PL CD8$^+$ cells were adoptively transferred together with H8 DC into C57BL/6 recipients. FACS staining was done as described in Figure 1. Data represent the mean +/- SD of 3 individual mice bled at day 7 after priming. This experiment was performed two times with similar results.

Discussion

T cell competition for antigen-expressing APCs has been suggested as a mechanism that can determine the selective outgrowth of higher affinity T cells (Busch and Pamer, 1999; Rees et al., 1999; Savage et al., 1999; Slifka and Whitton, 2001), but that can also play an important role in immunodominance. The experiments described in this study were designed to address the controversial issue of T cell competition for APC.

The feature of T cell competition has been addressed before, using different setups. The experimental system used by Perreault and colleagues (Loyer et al., 1999; Pion et al., 1999)is based on adoptively transferring male C57BL/6 (H-Y and B6^{dom1}), female C57BL/6 (B6^{dom1}) or male C3H.SW (H-Y) cells into female C3H.SW mice, followed by analysis of H-Y and of B6^{dom1}-specific CTL. B6^{dom1}-specific CTL were found to inhibit priming of H-Y-specific CTL when both epitopes were presented by the same APC. B6^{dom1}-specific CTL were shown to expand more rapidly, and eliminate APC before H-Y specific CTL

were primed, resulting in immunodominance of $B6^{dom1}$. $B6^{dom1}$-specific CTL may expand faster, because of more efficient priming; as it was shown in the same study, 800 $B6^{dom1}/D^b$ complexes and only 8 H-Y/D^b complexes were present on APC. The same mechanism was suggested to be operative in immunodominance in the C57BL/6-anti-BALB.B CTL response by Wolpert and colleagues. (Grufman et al., 1999; Wolpert et al., 1998): they reported that CTL response specific for the immunodominant epitope (H-28^c) developed faster that those specific for the subdominant epitopes (H-8^c, H-19^c and H-25^c).

To explain T cell competition, a second hypothesis has been suggested by Kedl, et al. (Kedl et al., 2000). They suggested that "crowding" of T cells of one specificity on the APC physically inhibited the access of other T cells to this APC and in addition excluded elimination of APC. This hypothesis received considerable attention recently. Kedl et al. transferred titrated numbers of TCR transgenic OT-1 cells (specific for OVA-derived S8L) together with infection with VV-OVA, and analyzed priming of endogenous S8L- and KVVRVDKL (K8L) (a subdominant, K^b-restricted OVA-derived epitope)-specific CTL. They found that OT-1 cells inhibited the priming of both endogenous responses. In addition, they primed mice with peptide-loaded DC (S8L and SIYRYYGL, the K^b-restricted 2C epitope) together with OT-1 transfer and found that priming of endogenous SIYRYYGL-specific CTL was inhibited if S8L was present on the same DC, but not if the two peptides were on different DC. They interpreted these data such that large numbers of OT-1 cells interacted with the S8LS-presenting APC, thus inhibiting priming of other CTL. As CTL precursors usually make up a rather small population in a naïve mouse that is dispersed over several lymphoid organs, it is difficult to conceive how this crowding should function under physiological conditions. Therefore, we used a comparable setup to further investigate the relevance of T cell competition for access to antigen expressing APC. We adoptively transferred TCR transgenic 318 cells together with priming with LCMV, with VV-G2 or with transgenic H8 DC , and analyzed the endogenous GP33 CTL response, as well as the response to two additional, LCMV glycoprotein-

derived epitopes (GP34 and GP276). We clearly could inhibit the endogenous GP33 CTL response by transfer of as few as 10^4 318 cells and completely inhibited the endogenous response by 10^5 or more 318 cells. This is in agreement with data published by others (Butz and Bevan, 1998; Kedl et al., 2000), and illustrates that there is apparently a limit to the number of specific T cells that can be primed or that can expand in a host (Butz and Bevan, 1998; Laouar and Crispe, 2000; Smith et al., 2000). This might be an important mechanism to disconnect precursor frequency and size of the immune response, thus allowing a response that is proportional to the antigenic load, as has been suggested before (Butz and Bevan, 1998; Smith et al., 2000). However, we could not reproduce the finding that T cells of one specificity inhibited priming of T cells of other specificities if these antigens were presented by the same APC; although the different ways of priming used by us led to responses of different magnitude (between 1.5-10% tetramer positive cells within the CD8 population), we never found inhibition of priming of the GP34 or of the GP276 response. Thus, in our experiments that were very similar to those published by Kedl, et al. (Kedl et al., 2000), we found no evidence for the hypothesis that CTL of one specificity can inhibit priming of CTL for another specificity by hampering access of the latter to the APC.

A possible explanation for this discrepancy might be that the OT-1 epitope (S8L) is presumably, due to its higher affinity for K^b, present in higher numbers on VV-OVA-infected APC than the subdominant epitope (K8L), resulting in more efficient (more and/or faster) priming of S8L-specific CTL, which was actually observed (0.4% S8L- and 0.07% K8L-specific CTL were primed by VV-OVA (Kedl et al., 2000). As a comparison, VV-G2 primes around 2% for all three GP-derived epitopes). Thus, before K8L-specific CTL were able to interact with sufficient antigen to be substantially primed, the APC might have been eliminated. Although no effect of transferred OT-1 cells on VV-OVA clearance was reported (Kedl et al., 2000), subtle effects on the number of APC, that are not reflected by reduction of virus titer may be sufficient to reduce the priming of the subdominant epitope. The discrepancy between the experiments using priming with DC might be explained by the

fact that Kedl et al. used peptide-loaded DC, whereas we used DC that produce the antigens endogenously. In our case, off-rates from the presenting MHC class I molecules do not confuse the system, and in addition, may be more physiological, as infected APC will also continuously present antigens.

Thus, our data do not support the hypothesis that T cell competition for access to antigen-expressing APC due to crowding (Kedl et al., 2000) is a phenomenon of functional importance in priming of antiviral immunity or in immunodominance. However, in some situations T cell competition has been shown to play a decisive role in immunodominance;in these studies, the mechanism has been shown to depend on differences in CTL priming and/or expansion as the major feature, resulting in elimination of APC by immunodominant CTL before the subdominant CTL could be substantially primed (Grufman et al., 1999; Loyer et al., 1999; Pion et al., 1999; Wolpert et al., 1998).

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Immunodominance of an Antiviral Cytotoxic T Cell Resonse Is Shaped by the Kinetics of Viral Protein Expression

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Summary

Lymphocytic choriomeningitis virus (LCMV) infection induces a protective CTL response consisting of glycoprotein (GP)- and nucleoprotein (NP)-specific CTL. We find that a small load of LCMV led to immunodominance of NP-CTL, whereas a large viral load resulted in dominance of GP-CTL. This is the first study describing that immunodominance is not fixed after infection with a given pathogen, but varies with the viral load instead. We assumed higher antigen sensitivity for NP-CTL, which would explain their preferential priming at low viral load, as well as their overstimulation resulting in selective exhaustion at high viral load. The higher antigen sensitivity of NP-CTL was due to faster kinetics of NP-epitope presentation. Thus, we uncover a novel factor that impinges upon immunodominance and is related to the kinetics of virus protein expression.

We propose that CTL against early viral proteins swiftly interfere with virus replication, resulting in efficient protection. If these "early" CTL fail in immediate virus control, they are activated in the face of higher viral load compared with "late" CTL and are therefore prone to be exhausted.

Thus, the observed absence of "early" CTL in persistent infections might not be the cause, but rather the consequence of viral persistence.

Introduction

The CD8+ cytotoxic T lymphocytes (CTL) recognize peptides presented by major histocompatibility complex (MHC) class I molecules on the surface of antigen presenting cells (APC) (Rammensee et al., 1993). These peptides are predominantly generated from endogenous proteins in a proteasome- and transporter associated with antigen processing (TAP)-dependent fashion (Groettrup et al., 1996; Momburg and Hammerling, 1998; Townsend et al., 1988). Viral peptides will therefore be presented on infected cells by MHC class I molecules, and as a result, virus-specific CTL are important effectors to control virus replication and spread (Guidotti et al., 1996; Kagi et al., 1994; Rickinson and Moss, 1997; Riddell et al., 1992; Zinkernagel and Doherty, 1979).

Infection of C57BL/6 (H-2^b) mice with LCMV induces a strong and protective CTL response that is dominated by four epitopes: glycoprotein (GP)-derived GP33-41/D^b, GP34-41/K^b, and GP276-286/D^b, and nucleoprotein (NP)-derived NP396-404/D^b. The response in BALB/c (H-2^d) mice is strongly dominated by NP118-126/L^d, but also responses to GP283-292/K^d are detectable (Blattman et al., 2000; Butz and Bevan, 1998; Gallimore et al., 1998c; Murali-Krishna et al., 1998; Sourdive et al., 1998; van der Most et al., 1998; van der Most et al., 1996; Weidt et al., 1998). After infection with LCMV-Armstrong (Arm), the CTL response in C57BL/6 mice is dominated by NP396-CTL (Blattman et al., 2000; Murali-Krishna et al., 1998), whereas infection of mice with faster replicating strains such as LCMV-WE, LCMV-Docile or LCMV-Arm Clone13 or -t1b (Zajac et al., 1998) results in immunodominance of GP33-CTL(Gallimore et al., 1998a; Gallimore et al., 1998c; Zajac et al., 1998).

Preferential priming of NP396-CTL at low virus load and immunodominance of GP33-CTL at high virus load suggest that NP396-CTL are more sensitive to antigen in some way, and selective exhaustion (Gallimore et al., 1998a; Oxenius et al., 1998; Zajac et al., 1998) of NP396-CTL may explain their gradual disappearance upon increasing viral load. Several studies described the association between decreased CTL responses and high viral load and disease progression in HIV infection(Klein et al., 1995; Ogg et al., 1998; Pantaleo et al., 1997) or infection with lymphocytic choriomeningitis virus (LCMV) (Moskophidis et al., 1993). Exhaustion of specific CTL in persistent infections is facilitated in the absence of CD4+ cells (Battegay et al., 1994; Cardin et al., 1996; Matloubian et al., 1994; Zajac et al., 1998), but can also happen in the presence of fully functional CD4+ cells (Oxenius et al., 1998).

We have performed experiments to delineate the relationship between virus-burden, immunodominance and protection against persistent infection. Other studies have previously identified differences in antigen processing, T cell repertoires, MHC/peptide/TCR avidities as important factors in controlling immunodominance. It was, however, clear from our early experiments that neither of these factors could account for the shifting pattern of immunodominance observed following infection of mice with viruses of

different replicative capacities. In fibroblasts that were infected with LCMV in vitro, the NP was detected substantially before GP by immunoprecipitation (Bruns et al., 1990). In this study, we show that this kinetic difference in protein production has consequences for LCMV-derived antigen processing and presentation: LCMV-infected cells presented NP-derived CTL epitopes 8 h before they presented GP-derived epitopes. NP-CTL indeed seemed to interact with LCMV-infected cells in vivo before GP-CTL, as we show that priming of GP-CTL in vivo was prevented by the presence of NP-CTL, but not vice versa. In addition, we show that selective expansion of NP-CTL at low viral load is a feature intrinsic to LCMV, as it did not occur after infection with recombinant vaccinia virus carrying LCMV CTL epitopes.

We propose that the kinetic difference in antigen presentation explains the preferential priming of NP-CTL at low viral load, their selective exhaustion at high viral load as well as their relatively high protective capacity in vivo (Gallimore et al., 1998a). Thus, we discovered a novel factor that impinges on immunodominance and which is related to the kinetics of antigen presentation.

Materials and Methods

Mice

C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were bred in the Institut für Labortierkunde, University of Zürich (Zürich, Switzerland). 318 TCR transgenic mice expressing the P14 TCR that is specific for LCMV GP33-41/D^b are on a C57BL/6 background (Pircher et al., 1990). CB6F1 (H-2^{bxd}) were obtained from Charles River Breeding Laboratories (Hannover, Germany). DIETER mice have a pure C57BL/6 background and expression of GP33-41 and NP396-404 as a transgene can be induced in their DC (Probst et al., 2003a). All mice were kept under specific pathogen free conditions and were at least 6 weeks old at the beginning of the experiments. Animal experiments were performed in compliance with Swiss national and cantonal laws (Kantonales Veterinäramt Zürich) on animal protection.

Viruses

LCMV-WE was originally obtained from Dr. F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) (Lehmann-Grube, 1971), LCMV-Armstrong was obtained from Dr. M. Oldstone (The Scripps Clinic and Research Foundation, La Jolla, CA)(Buchmeier et al., 1980) and LCMV-Docile (LCMV-Doc) was a variant isolated from an LCMV-WE carrier mouse and was obtained from C. Pfau (Departement of Biology, Rensselaer Polytechnic Institute, Washington, DC) (Pfau et al., 1982). LCMV was propagated on L929 cells at a low multiplicity of infection (m.o.i.). Vaccinia Virus (VV), strain WR, was originally obtained from Dr. B. Moss (National Institutes of Health, Bethesda, MD). rVV carrying one of the two immunodominant CTL epitopes of LCMV in the context of H-2^b were generated by cloning synthetic oligonucleotides encoding for the peptide sequence (5'-CATG-xxx-xxx-xxx-TA-3' and 5'-CTAGAT-xxx-xxx-xxx-3', where xxx represents the peptide coding sequence) into the NcoI/BglII-digested transfer vector pSC11.3OR2 (Cerundolo et al., 1995). The minigene sequences were: GP33=AAAGCTGTGTACAATTTCGCCACCTGT (MKAVYNFATC), and NP396=TTTCAACCACAAAATGGGCAATTCATA (MFQPQNGQFI). Recombinant viruses were selected using bromodeoxyuridine and recombinant plaques were identified by β-galactosidase activity as described (Moss, 1998). Recombinant VV were plaque-purified three times on BSC40 cells. As a control, VV expressing the Vesicular Stomatitis Virus glycoprotein (VVc) was used. All VV were propagated at a low multiplicity of infection (m.o.i.) on BCS-40 cells.

Dendritic Cells

Bone marrow derived dendritic cells (DC) were generated from the femora and tibiae of DIETER mice as previously described (Inaba et al., 1992). Mice were primed by i.v. injection of $5x10^5$ DC.

Cell Lines

MC57G are methylcholanthrene-induced fibrosarcoma cells of C57BL/6 origin. EL-4 are dimethylbenzanthrene-induced thymoma cells of C57BL/6 origin. P815 are mastocytoma cells from BALB/c origin. BSC-40 cells are a

subclone of the green African monkey kidney cell line BSC-1. NP118-126/L^d-specific and GP34-41/K^b-specific T cell hybridomas carrying the LacZ reporter constructed were a kind gift of Marcus Groettrup (University of Constance, Konstanz, Germany). The NP118 hybridoma recognizes NP118-126 (RPQASGVYM) plus H-2L^d, the GP34 hybridoma GP34-41 (AVYNFATC) plus H-2K^b (Schwarz et al., 2000; Shastri and Gonzalez, 1993).

Peptide-specific CTL lines

Splenocytes from LCMV-WE memory mice (injected with 100 pfu LCMV-WE at least 2 months before) were restimulated with irradiated and peptide-loaded (10^{-8} M), thioglycollate-elicited macrophages (1 ml thioglycollate i.p. at d –3) at a ratio of 20:1 in the presence of 25 U/ml IL-2. Cultures were restimulated every week as above at a ratio of 5:1. After 3 rounds of restimulation, CTL were found to be of single specificity. BALB/c memory CTL were restimulated with NP118-126 (RPQASGVYM, L^d) or with GP283-292 (GYCLTKWMIL, K^d), C57BL/6 memory CTL were restimulated with GP33-41 (KAVYNFATC, D^b), GP276-286 (SGVENPGGYCL, D^b) or with NP396-404 (FQPQNGQFI, D^b).

CTL assays

In order to measure direct ex vivo LCMV-specific CTL responses, splenocytes were tested for cytolytic activity towards peptide-loaded (10^{-7} M) or unloaded, ^{51}Cr-labeled EL-4 or P815 target cells in a 5 h assay. The CTL activity of LCMV-specific memory CTL was measured 5 d after in vitro restimulation of splenocytes with peptide-loaded (10^{-8} M), irradiated, syngeneic thioglycollate macrophages (responder:stimulator=20:1) in the presence of 25 U/ml IL-2. Standard dilutions of cultures were tested for cytolytic activity on peptide-loaded, ^{51}Cr-labeled EL-4 or P815 cells in a 5 h assay.

Staining with tetrameric MHC class I-peptide complexes

Tetrameric complexes containing biotinylated H-2D^b or H-2L^d, β_2-microglobulin, the relevant peptide and extravidin-PE were generated as described (Altman et al., 1996; Gallimore et al., 1998b). Approximately 5 x 10^5 cells were stained with 0.5-1 μg of tetramer in 25 μl FACS Buffer (FB, PBS + 2% FCS + 0.01% NaN_3 + 20 mM EDTA) at 37°C for 10 minutes. One μl of anti-CD8α-FITC (clone 53-6.7) was added and staining was continued for 30

minutes at 4°C. Cells were washed three times, fixed (FACS Lysis Solution, Becton Dickinson, Mountain View, CA) and were analysed by flow cytometry (FACS-SCAN, Beckton-Dickinson, Mountain View, CA) using CellQuest software. For tetramer dissociation assays, cells were stained with tetramer as above, anti-CD8α-FITC, anti-CD4-biotin, anti-B220-biotin, anti-I-A^b-biotin, for 30 minutes at 4°C, were washed once, were stained with streptavidin-CyChrome for 20 minutes at 4°C (all PharMingen) and were washed three times. Cells were suspended in 0.5 ml FACS buffer containing propidiumiodide and 150 μg/ml of mouse monoclonal IgG2a anti-D^b (T21-460, obtained from Günter Hämmerling, DKFZ, Heidelberg, Germany) to prevent rebinding of dissociated tetramer at t=0. Samples were incubated on ice for the indicated times and tetramer staining was analysed on CD8+ve, CD4-ve, B220-ve, I-A^b-ve, propidiumiodide-ve cells. We measured the rate of decay using flow cytometry and we obtained linear decay plots of the natural logarithm of the normalized fluorescence versus time, indicating that tetramer dissociation was occurring stochastically and that the resulting tetramer staining half-lives should be proportional to the half-lives of the respective TCR-peptide/ MHC complexes (Savage et al., 1999).

Intracellular Cytokine Staining for Interferon-γ (ICS)

One million splenocytes were incubated in 200 μl IMDM + 10% FCS and antibiotics for 6 h at 37°C with 10^{-6}M of the specific peptide or with medium alone as a negative control. To enhance intracellular accumulation of IFNγ, Brefeldin A was added at a final concentration of 5 μg/ml for the whole duration of the culture. For staining, cells were put at 4°C, washed with FACS buffer (FB, see above) and stained with anti-CD8β-PE (PharMingen) for 30 minutes at 4°C. Cells were washed twice with FB and fixed with 100 μl of 4% paraformaldehyde in PBS for 5 minutes at 4°C. Two ml of permeabilization buffer (PB: FB + 0.1% w/v saponin) were added and cells were incubated for 10 minutes at 4°. Cells were spun down and stained intracellularly with anti-mouse-IFNγ-FITC (clone AN18, PharMingen) in PB for >60 minutes at 4°C. After three washes with PB, cells were resuspended in FB and analysed by flow cytometry as described above.

Kinetics of antigen presentation by LCMV-infected CB6F1 macrophages

Thioglycollate-elicited macrophages were isolated and infected with LCMV-Arm or with LCMV-WE at an m.o.i. of 2 and were further incubated at 37°C for the indicated times. Infected macrophages were harvested (t=0) and Brefeldin A was added to a final concentration of 5 μg/ml to freeze cells in their state of antigen presentation. The degree of infection was measured by intracellular staining for LCMV-NP, using FITC-labelled monoclonal rat-anti-LCMV NP (VL4 (Battegay et al., 1991b)), followed by FACS analysis. Macrophages of the same batch were used as targets in a chromium release assay and as stimulators in ICS using peptide-specific CTL lines as effectors/responders. For these experiments, part of the macrophages were labelled with ^{51}Cr (in the presence of Brefeldin A) and were incubated with titrated numbers of peptide-specific CTL in a 5 h chromium-release assay in the presence of Brefeldin A. Another part of the macrophages was used as stimulators of IFNγ production in a 6 h ICS in the presence of Brefeldin A ($3x10^5$ macrophages plus $3x10^5$ tetramer-positive CTL) as described above. In the chromium-release assay as well as in ICS, macrophages loaded with titrated amounts of each of the five peptides (10^{-6}-10^{-12}M) were included as a sensitivity control for the individual CTL lines.

Kinetics of antigen presentation by cells from LCMV-infected mice

CB6F1 mice were infected i.v. with 2 x 10^7 pfu of LCMV-WE. The high inoculum size was chosen to ensure maximal numbers of infected cells in the spleen. At defined time points after infection, spleens were homogenized and used as antigen presenting cells. Five x 10^4 T cell hybridoma cells were incubated overnight in 96-well round bottom plates at 37°C with 1.5 x 10^5 splenocytes from infected CB6F1 mice or with 1.5 x 10^5 naïve CB6F1 splenocytes with titrated amounts of the relevant antigenic peptide in 200 μl medium containing 5 μg/ml Brefeldin A to freeze their condition of antigen presentation. After incubation, cultures were processed as described previously (Usherwood et al., 1999). Briefly, cells were washed with PBS and were subsequently fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 5 min at 4°C. The plates were washed with PBS and

overlaid with 50 μl of staining solution (1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2mM $MgCl_2$ in PBS). T cell hybridomas that were stimulated through the TCR can be visualized by blue staining, resulting from a LacZ reporter gene under control of the IL-2 promoter/enhancer (Shastri and Gonzalez, 1993). Triplicate cultures were microscopically examined, and the number of blue cells per well was counted after overnight incubation at 37°C.

Measurement of virus titers

LCMV titers were determined in the spleens and livers at indicated time points after infection. To determine LCMV titers, organs were removed, homogenized in MEM/2% FCS and titrated by tenfold dilution onto monolayers of MC57G cells in 24 well plates. LCMV was detected after 2 d incubation at 37°C by an immunofocus assay using an LCMV NP-specific monoclonal antibody (VL4), as previously described (Battegay et al., 1991b). The detection limit of this focus forming assay is 100 pfu/ml.

Results

Immunodominance is influenced by the LCMV inoculum, and is not related to T cell intrinsic features such as TCR affinity or the capacity to expand

As LCMV is a noncytopathic virus that replicates massively in its natural host, the mouse, simple titration of the inoculum does not cover a wide range of viral loads. To circumvent this problem and to be able to analyse immunodominance at gradually increasing viral loads, we took advantage of different viral isolates, that are known to have different replication rates, such that LCMV-Arm < LCMV-WE < LCMV-Doc. We infected C57BL/6 mice with titrated (10-fold dilutions) amounts of different LCMV strains and measured virus titres 5 d later in the spleen. We then chose the inoculum size to analyse immunodominance such that we had a graded increase in viral load (see Figure 1). We infected C57BL/6 mice with 10^2 or 10^6 pfu of LCMV-Arm, with 10^0, 10^2 or 10^6 pfu of LCMV-WE or with 10^2 of 10^6 pfu of LCMV-Doc. The viral

load was measured in spleen at d 6, d 8 and d 15 (Figure 1A, *upper panels*) and liver (comparable to spleen, data not shown), and was found to increase in the following order: 10^2 pfu LCMV-Arm < 10^6 pfu LCMV-Arm < 10^0 pfu LCMV-WE < 10^2 pfu LCMV-WE < 10^2 pfu LCMV-Doc < 10^6 pfu LCMV-WE < 10^6 pfu LCMV-Doc. Virus was not detectable anymore at d 50 in any of the mice, except for mice infected with 10^6 pfu LCMV-Doc (50'000 pfu/spleen), which is in accordance with previously published findings (Moskophidis et al., 1993). Tetramer staining 15 d after infection (Figure 1A, middle panels) showed clear immunodominance of NP396-CTL at low viral load, which gradually decreased with increasing viral load. This resulted in immunodominance of GP33-CTL at higher viral loads. Functional analysis of the CTL by ICS ex vivo at d 15 after infection made the observed difference even more clear (Figure 1A, *lower panels*): At low viral load, NP396-CTL were immunodominant, whereas they were functionally undetectable (although physically present to some extent) after infection with 10^6 pfu LCMV-WE. In contrast, the number of GP33-CTL increased at increasing viral load. High dose (10^6 pfu) LCMV-Docile resulted such high amounts of rapidly spreading virus, that also GP-specific CTL were functionally and physically exhausted (Gallimore et al., 1998b; Moskophidis et al., 1993; Ou et al., 2001), ultimately resulting in lifelong virus persistence. The apparent higher sensitivity to exhaustion of NP-CTL might be explained by a lower naïve precursor CTL (CTL_p) frequency of NP-CTL compared with GP-CTL; however, this possibility cannot account for the preferential priming of NP-CTL at low virus load. The viral loads after infection with 10^6 pfu LCMV-Arm and with 1 pfu LCMV-WE were apparently similar (Figure 1A, *upper panels*), however, the immunodominance profile was found to be different (Figure 1A, *middle and lower panels*). This is possibly due to the fact that the resolution of the plaque assay is not sufficient to detect small, but probably biologically relevant differences in viral load immediately after infection. Moreover, analysis of the GP- and NP-CTL response after infection of CB6F1 mice with 10^2 pfu LCMV-Arm, 10^2 or 10^6 pfu LCMV-WE demonstrated that the NP396- and the NP118-specific response behaved similarly (i.e. going down upon increasing virus

load), and opposite to the GP33-specific response (i.e. increasing upon higher virus load) (Figure 1B). Because the NP118 response is a very strong one, selective exhaustion of NP-CTL at high virus load is unlikely to result from low CTL_p frequencies. The preferential priming of NP-CTL at low viral load and their exhaustion upon increasing antigen levels suggests that NP-CTL have somehow a higher sensitivity to antigen than GP-CTL.

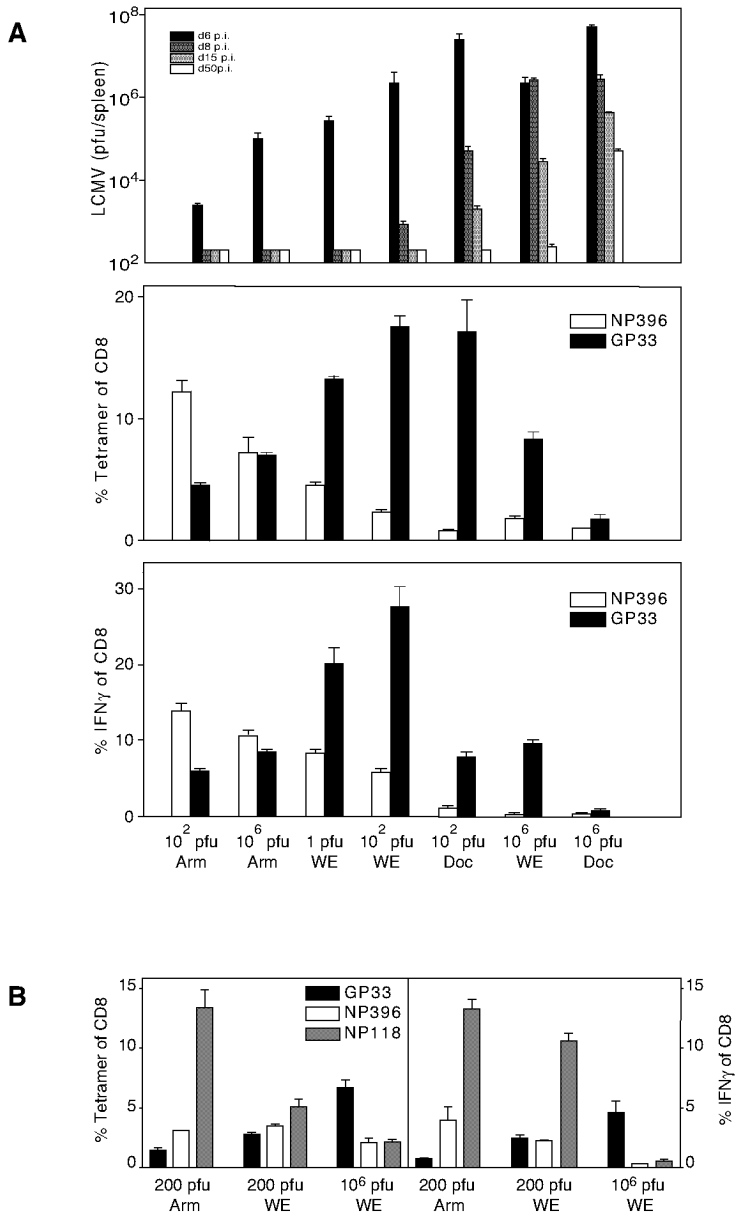


Figure 1: NP396-specific CTL are preferentially primed under conditions of low viral load, and are gradually exhausted as viral load increases.

A, C57BL/6 mice were infected with 10^0, 10^2 or 10^6 pfu of LCMV-Armstrong, LCMV-WE or LCMV-Doc. *Upper panels*: Viral titers were measured in the spleen at indicated time points after infection. *Middle panels*: The physical presence of GP33- and NP396-CTL in the spleen was measured at d 15 after infection by tetramers. *Lower panels*: The functional presence of GP33- and NP396-CTL in the spleen was measured at d 15 after infection by ICS. The values are the mean of 3 mice, one representative experiment of 4 is shown. **B**, CB6F1 mice were infected with 10^2 pfu LCMV-Arm, 10^2 or 10^6 pfu LCMV-WE. *Left panel*: The physical presence of GP33-, NP396- and NP118-CTL in blood was measured at d 29 after infection by tetramers. *Right panel*: The functional presence in the spleen was measured at d 29 after infection by ICS. The values represent the mean of two mice, one experiment of two is shown. Black bars: GP33; White bars; NP396; Grey bars: NP118

We considered the possibility that CTL bearing TCR with high affinity were preferentially activated at low virus load and were more likely to be over-stimulated and exhausted (Moskophidis et al., 1993; Zajac et al., 1998) at higher virus load. As a consequence, we had to assume that NP396-CTL generally have a TCR of higher affinity compared with GP33-CTL. The most reliable manner to determine TCR affinities is probably by BiaCore, however, this method cannot be applied on polyclonal T cell populations. Therefore, we used tetramer dissociation on ex vivo prepared CTL as a measure for the TCR affinities of polyclonal, but mono-specific CTL populations after infection with LCMV (Savage et al., 1999). We always included 318 TCR transgenic T cells that are specific for GP33/D^b (Pircher et al., 1990) as a reproducibility control. We found comparable tetramer dissociation kinetics of GP33-CTL and of NP396-CTL after infection with LCMV (Figure 2A and 2B), suggesting no apparent difference in TCR affinity between the two specificities. In addition, we found no difference in TCR affinities between infection with LCMV-WE and -Arm (Figure 2A and 2B). Also by titrating peptides in intracellular cytokine stain for IFNγ (ICS) or on targets in a CTL assay, we did not find substantial differences between GP33-41 and NP396-404 (data not shown), provided that in these particular experiments we used the optimized D^b binder KAVYNFATM, with an affinity for D^b comparable to NP396-404, instead of the natural peptide KAVYNFATC. In addition, when we used the natural peptide (KAVYNFATC) and corrected for lower affinity to D^b (van der Most et al., 1998), we also did not see differences in peptide titrations. The interaction of the TCR with H-2D^b/GP33-41 is not affected if the naturally occurring cystein on position 9 is replaced by a methionin (Achour et al., 2002). Thus, the change of immunodominance after infection with different amounts of virus can't be explained by differences in TCR affinity of polyclonal NP396- and GP33-CTL populations.

To distinguish between factors intrinsic to the responding CTL populations and LCMV-specific features, we primed mice with $5x10^5$ bone marrow derived DIETER DC transgenic for GP33-41 and NP396-404 (Probst et al., 2003a), and challenged them 10 d later with $2x10^6$ pfu of recombinant vaccinia virus

(VV) expressing GP33-41 or NP396-404 as a minigene (VV33, VV396) or with 100 pfu LCMV-WE. We found that GP33-CTL and NP396-CTL expanded to a similar extent after VVminigene challenge (Figure 2C). In contrast, NP396-CTL expanded considerably stronger than GP33-CTL after LCMV challenge. This indicated that the difference in expansion seen in LCMV-challenged mice is not an intrinsic property of the individual CTL populations but rather depends on LCMV as such.

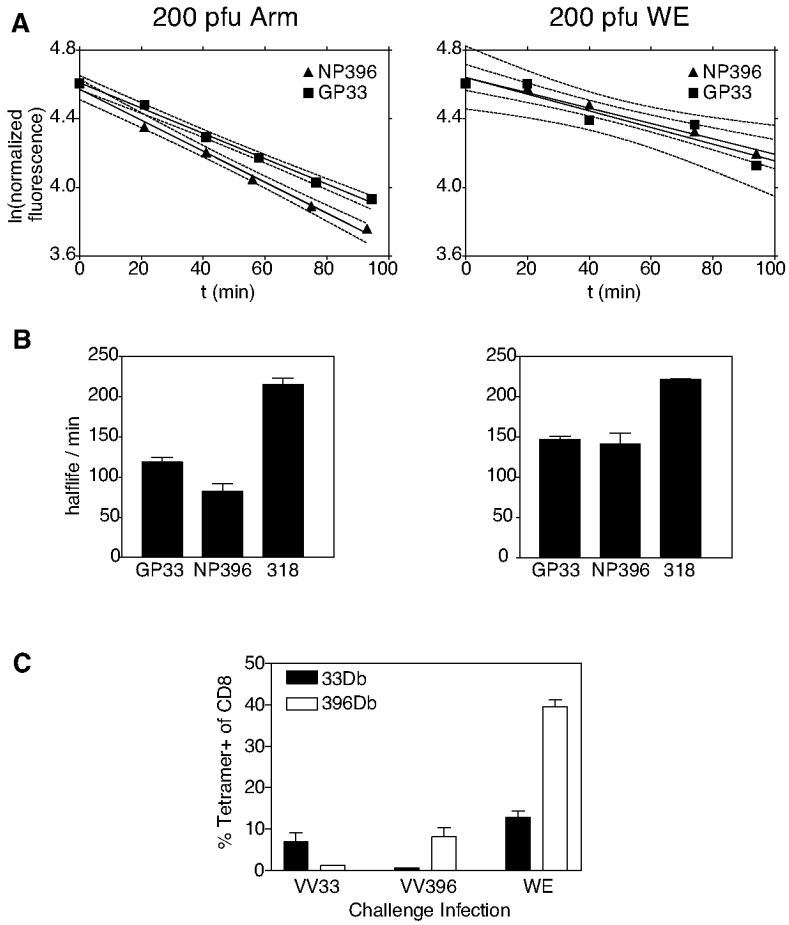


Figure 2: GP33- and NP396-specific CTL show similar TCR affinity as measured by tetramer dissociation rates and are not intrinsically different with respect to their ability to expand in vivo.

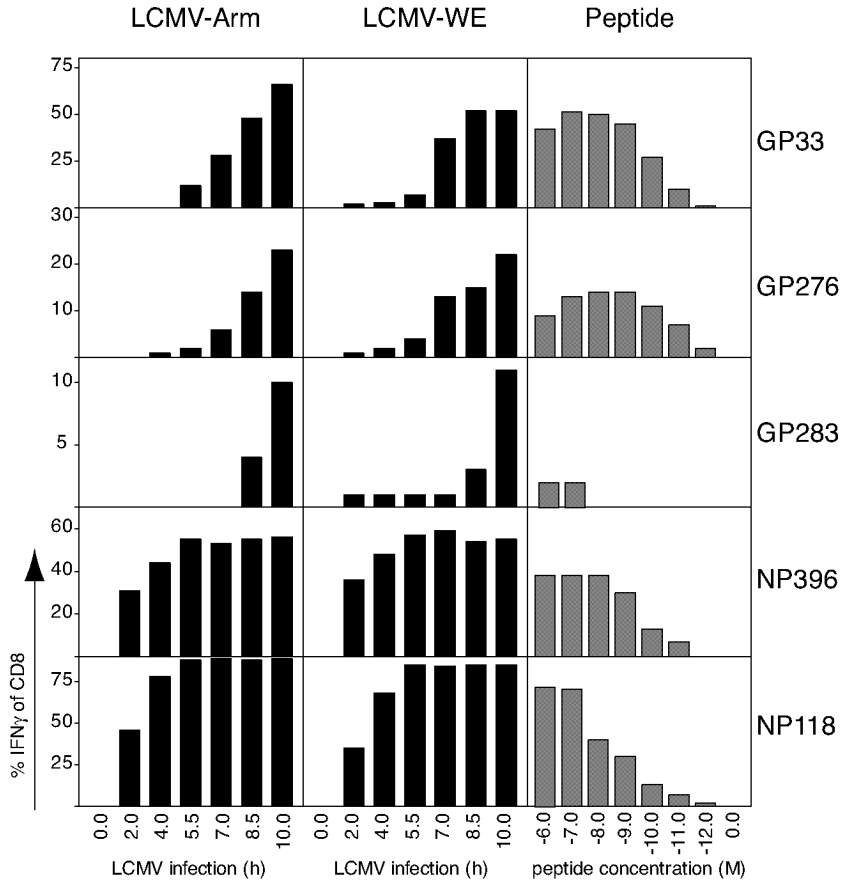
C57BL/6 mice were infected i.v. with 10^2 pfu LCMV-Armstrong or –WE, and splenocytes were stained with tetramer as described in *Materials and Methods* 35 d later. Tetramer-stained cells were incubated on ice for indicated times in the presence of saturating amounts of a monoclonal anti-D^b antibody in order to prevent rebinding of dissociated tetramer as described (Savage et al., 1999). The dissociation of tetramers was measured by FACS.

A, Representative decay plots of one LCMV-Arm and of one LCMV-WE infected mouse. Decay plots show the natural logarithm of the normalized fluorescence versus time. B, The mean of calculated half lives of TCR-peptide/H-$2D^b$ interaction of 3 individual mice is plotted. C, C57BL/6 mice were primed with $5x10^5$ syngeneic DIETER DC transgenic for GP33 and NP396 (Probst et al., 2003a), and were challenged 10 d later with $2x10^6$ pfu VV33, $2x10^6$ pfu VV396 or with 10^2 pfu LCMV-WE. Expansion of GP33- and of NP396-CTL was measured in the blood by tetramers 8 d after challenge. Results represent the mean of two individual mice, and one representative experiment of two is shown.

LCMV-infected macrophages present LCMV NP-derived CTL epitopes with increased kinetics compared with LCMV GP-derived CTL epitopes

Using infected cells in vitro, it has been shown that the LCMV NP is detectable at least 12 h before LCMV GP on the protein level (Bruns et al., 1990). Thus, NP-derived epitopes can be presented before GP-derived epitopes, which may have important consequences for CTL induction: NP-CTL will be primed before GP-CTL, and they will thus start controlling the virus before most GP-specific precursors had the chance to interact with their nominal peptide on LCMV-infected cells. Therefore, NP-CTL generally will be primed in the face of higher viral load than GP-CTL and can interfere with priming of GP-CTL at the same time. We investigated the existence of kinetic differences in the onset of presentation between GP-and NP-derived epitopes by infecting thioglycollate-elicited CB6F1 ($H\text{-}2^{bxd}$) macrophages with LCMV-Arm or –WE at an m.o.i. of 2, resulting in infection of 100% of the macrophages as demonstrated by intracellular staining with an monoclonal antibody against LCMV NP (VL4 (Battegay et al., 1991a)). Subsequently, their status of antigen presentation was frozen at different time points after infection by Brefeldin A and these macrophages were used as stimulators for IFNγ production (ICS) by mono-specific CTL lines, specific for 3 GP-derived (GP33-41/D^b, GP276-286/D^b, GP283-292/K^d) and 2 NP-derived (NP118-126/L^d, NP396-404/D^b) epitopes. We found that GP-derived epitopes were detectable by CTL after 7-8 h of infection and reached their maximum after 10 h, whereas NP-derived epitopes reached their maximum around 2 h after infection (Figure 3). This was true for both LCMV-Arm (Figure 3, left panels) and –WE (Figure 3, *middle panels*), excluding the possibility that differences in immunodominance seen in vivo after infection with LCMV-Arm (NP396 immunodominant) or –WE (GP33 immunodominant) were due to differences in antigen processing or presentation between the two LCMV strains. Because differences in antigen sensitivity between the individual CTL lines would obscure our data, we included CB6F1 macrophages loaded with titrated amounts of peptide in the same experiment to check the antigen sensitivity of CTL lines. Half maximal stimulation of IFNγ production required

10^{-10}M of GP33, 10^{-11}M of GP276, between 10^{-9} and 10^{-10}M of NP396 and 10^{-8}M of NP118 (Figure 3, *right panels*). This indicates that GP-CTL lines were slightly more sensitive to antigen compared with the NP-CTL lines, excluding that faster detection of NP epitopes was due to higher antigen sensitivity of NP-CTL lines. However, the GP283-CTL line appeared relatively antigen insensitive (>10^{-7}M required), and GP283-292 also became detectable extremely late (8.5 h) after infection (Figure 3). This may be explained by the fact that CB6F1 macrophages express limited numbers of the GP283-292 presenting H-2K^d molecule, whereas substantially higher numbers of H-2D^b and H-2L^d are expressed (data not shown). Using the same macrophages (infected for different times with LCMV-Arm or –WE or loaded with titrated peptide) as targets for peptide-specific CTL lines in a 5 h ^{51}Cr-release assay, the abovementioned kinetic differences were confirmed: NP-CTL lines lysed LCMV-infected macrophages after 2 h of infection (the earliest time point studied), whereas GP-CTL started lysing the same targets after 8-10 h (data not shown).



FFigure 3: NP-derived CTL epitopes are presented to CTL with faster kinetics than GP derived CTL Epitopes.

Thioglycollate-elicited CB6F1 macrophages were infected with LCMV-Armstrong (*left panels*), or with LCMV-WE (*middle panels*) with an m.o.i. of 2, and the state of antigen presentation was frozen at indicated time points by addition of Brefeldin A (5 μg/ml). Subsequently, these macrophages were used to induce IFNγ production by 5 independent, peptide-specific CTL lines as a read out. CTL were incubated with infected macrophages in the presence of Brefeldin A for 6 h, and were surface stained for CD8β and intracellularly for IFNγ. *Right panels* The same CTL lines were incubated as described above with CB6F1 macrophages loaded with titrated amounts of peptide to determine the peptide sensitivity of the individual CTL lines. One representative experiment of four is shown.

LCMV-infected splenocytes ex vivo present LCMV NP-derived CTL epitopes with increased kinetics compared with LCMV GP-derived CTL epitopes

LCMV is known to infect a variety of cells in vivo, such as dendritic cells and macrophages (Sevilla et al., 2000), and therefore, in vitro infected, thioglycollate-elicited macrophages may not represent the physiologically

relevant population presenting LCMV-derived epitopes in vivo. In order to analyze the kinetics of antigen presentation in vivo, we infected CB6F1 mice with 2 x 10^7 pfu LCMV-WE and used their splenocytes at different time points after infection as APC for NP118-126/L^d or GP34-41/K^b specific T cell hybridomas (Schwarz et al., 2000; Usherwood et al., 1999). To exclude the possibility that differences in antigen-sensitivity between the two hybridomas would influence our result, we included uninfected CB6F1 macrophages with titrated amounts of peptide in the same experiment. For both hybridomas maximal numbers of blue cells were found at 10^{-7}M peptide or higher and background numbers were found below 10^{-10}M of peptide. This shows that the GP34/K^b-specific and the NP118/L^d-specific hybridoma were equally sensitive to antigen.

The kinetics of antigen presentation by in vivo infected splenocytes were similar to those by in vitro infected, thioglycollate-elicited macrophages (Figure 4). GP34-specific T cells were activated by splenocytes that were infected for 12 h or more, and splenocyte infection for 20 h or more induced maximal numbers. In contrast, infection of splenocytes for 4 h or more resulted in the presentation of the NP118 epitope and maximal levels were reached after 12 h. The maximal numbers of activated hybridomas after incubation with infected splenocytes were about 50% of the maximal values observed after incubation with peptide in both cases. This may be explained by the fact that peptides were present throughout the overnight culture, thus avoiding the influence of peptide off-rates as is the case with infected splenocytes in the presence of Brefeldin A.

Presentation by in vivo infected splenocytes displayed a delay of about 2 h for both epitopes (NP- and GP-derived) compared with the in vitro infected macrophages (Figure 3). This may be explained by the lower peptide sensitivity of the hybridomas (about 10^{-8}M) compared to the mono-specific CTL lines we used (Figure 3), or by the fact that it takes LCMV slightly longer to infect cells in vvo than in vitro, where the cells were infected at a high m.o.i. and in very close contact with the virus.

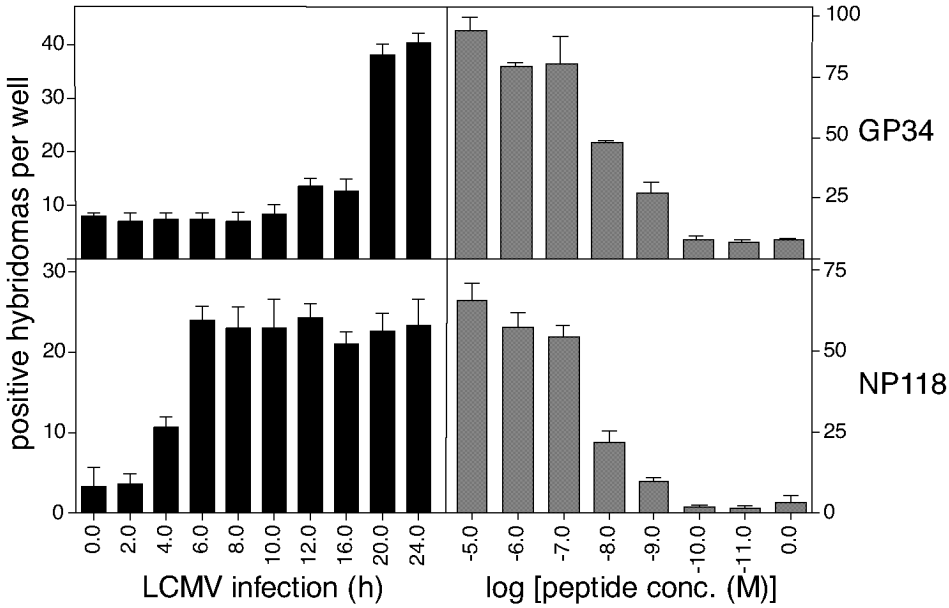


Figure 4: LCMV-infected splenocytes ex vivo present LCMV NP-derived CTL epitopes with Increased kinetics compared with LCMV GP-derived CTL epitopes.

CB6F1 mice were infected i.v. with 2 x 10^7 pfu LCMV-WE, their splenocytes were isolated at different time points after infection and were used as antigen presenting cells for NP118-126/L^d or for GP34-41/K^b specific T cell hybridomas. 5x10^4 T cell hybridomas were incubated overnight at 37°C with 1.5x10^5 LCMV-infected splenocytes (*left panels*) or with 1.5 x 10^5 uninfected splenocytes in the presence of titrated amounts of peptide (*right panels*). Five µg/ml Brefeldin A was present throughout the culture period to freeze the status of antigen presentation by the infected splenocytes. After stimulation through the T cell receptor, T cell hybridomas can be visualized by blue staining, resulting from a LacZ reporter gene under control of the IL-2 promoter/enhancer (Shastri and Gonzalez, 1993). Cultures were microscopically examined, and the number of blue cells per well was counted. Values represent the mean ± SD of Triplicate cultures. One mouse was used per time point of infection. One representative experiment out of two is shown.

Kinetic differences in the presentation of LCMV-derived CTL epitopes can be visualised in vivo

To show the relevance of faster kinetics of presentation of NP-derived epitopes in vivo, we designed the following experiment: We reasoned that "early" memory CTL (e.g. NP-CTL) recognize infected cells and start to eliminate them before "late" CTL (e.g. GP-CTL) had the chance to interact with their antigen, and thus substantially interfere with the priming of the latter. As a consequence, "early" memory CTL (e.g. NP-CTL) should block priming

of “late” CTL (e.g. GP-CTL) upon infection with LCMV, but not vice versa. Thus, we primed mice with VV33, VV396 or VVc and challenged them 30 d later with 100 pfu LCMV-WE to investigate whether we could prime GP-CTL in the face of a pre-existing NP-CTL memory response and vice versa. It is important to note that priming with VV33 or with VV396 protects mice against subsequent LCMV challenge, resulting in very low (undetectable) LCMV levels (data not shown). We measured the frequency of GP33- and NP396-CTL in the spleen by tetramer staining (Figure 5) at d 9 after LCMV challenge, and confirmed the data by a primary ex vivo CTL assay 9 d after LCMV infection (data not shown).

After VV396 priming, pre-existing memory NP396-CTL were found to expand massively upon LCMV challenge (Figure 5). GP33-CTL were not primed by LCMV in the presence of memory NP396-CTL (Figure 5). This can be explained by different kinetics of antigen presentation: NP396-CTL could start to control LCMV-infected cells before GP33-CTL could efficiently interact with them, resulting in inhibition of GP33-CTL priming. In the reverse situation, VV33 priming results in enhanced expansion of GP33-CTL after LCMV challenge compared with VVc priming, thus demonstrating the increased number of GP33-specific precursors due to VV33 priming (Figure 5). Interestingly, and in contrast with the situation described above, pre-existing memory GP33-CTL did not interfere with the priming of NP396-CTL (compare VVc and VV33 in Figure 5). This shows that GP33-CTL, even although present as memory cells and at increased CTL_p numbers, could not interfere with the priming of NP396-CTL. Because GP is presented with delayed kinetics compared with NP, GP33-CTL were too late to interfere with NP396-CTL priming. The ex vivo CTL assay performed on d 9 confirmed the tetramer data (data not shown). It is unlikely that differences in priming between VV33 and VV396 are responsible for the observed findings: Priming with VV33 or with VV396 resulted in a specific CTL response of similar size as determined by tetramer analysis (0.8-1.0% of the CD8+ at d 10 after infection) and by limiting dilution analysis (1 in 10^4 splenocytes at d 7 after infection). The frequency of specific memory CTL was comparable for VV33- and VV396-

infected mice over at least 5 weeks as measured by protection against LCMV challenge (data not shown).

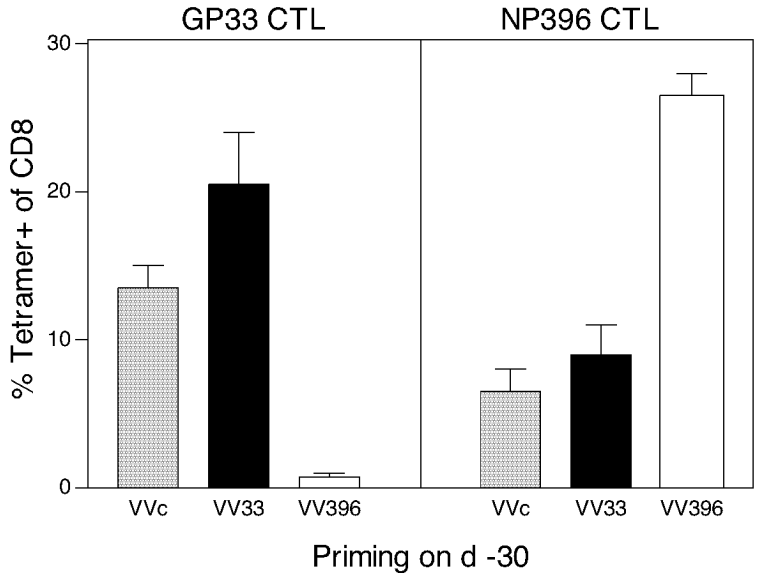


Figure 5: NP396-specific memory inhibits priming of GP33-specific cells, but not vice versa

C57BL/6 mice were primed by i.p. injection of $2x10^6$ pfu rVV33, rVV396 or rVVc, and challenged 30 days later with 10^2 pfu LCMV-WE. The expansion of GP33- and NP396-CTL was measured in the blood by tetramer staining after 9 d. Results represent the mean of three individual mice. One representative experiment of three is shown.

Discussion

LCMV infection induces a protective CTL response against both GP- and NP-derived epitopes. We describe here that inocula leading to a relatively low virus load (e.g. LCMV-Arm) resulted in immunodominance of NP-CTL, whereas increasing viral load (e.g. LCMV-WE or –Doc) coincided with decreasing NP-CTL, ultimately resulting in immunodominance of GP-CTL. We explained this finding by a higher sensitivity to antigen of NP-CTL compared with GP-CTL: NP-CTL thus would be preferentially activated by low levels of antigen, and (partially) exhausted (Moskophidis et al., 1993; Ou et al., 2001; Zajac et al., 1998)(18,20,44) upon increasing viral load. It has been suggested (Sevilla et al., 2000) that different strains of LCMV infect different cells in vivo: this would preclude comparison between strains as we do here. We infected

C57BL/6 mice with 10^6 pfu of LCMV-Arm, -WE or –Doc, and stained splenocytes for subset markers and LCMV-NP. We found that, independent of the strain used to infect, 5-8% of CD11c+, 0.7-1.5% of CD11b+ and <0.5% of CD19+ or TCR$\alpha\beta$+ cells stained positive for LCMV-NP. This excludes that changes in immunodominance were due to different tropism of the individual strains. Comparison of the GP33- and the NP396-specific CTL response after infection with 1 or with 100 pfu LCMV-WE demonstrates that the GP33-specific response increased, whereas the NP396-response decreased with higher viral load (Figure 1). The data of Ou et al. (Ou et al., 2001) can be interpreted along the same line: They showed that infection of mice deficient for the IFN$\alpha\beta$ receptor (A129 mice) with 100 pfu LCMV-WE or with 10^5 pfu LCMV-Arm resulted in a substantially higher viral load and in selective exhaustion of NP396 CTL when compared with wild type control mice. Thus, also without varying the virus strain/dose used for infection, but by choosing the conditions such that the virus replicates to higher titers, NP-CTL were found to gradually disappear. We considered that the higher antigen sensitivity of NP-CTL could be due to 1) higher TCR affinity of NP-CTL, 2) prolonged presentation of NP compared with GP by infected cells (Oldstone and Buchmeier, 1982), or 3) different kinetics of presentation of NP- vs. GP-epitopes (Bruns et al., 1990). We found no difference in TCR affinity as measured by tetramer dissociation (Savage et al., 1999) between NP396- and GP33-CTL, or by peptide titration in CTL assay and in ICS. The finding that in mice persistently infected with LCMV, GP expression was not found after d 15, whereas NP could be detected in all mice even after 80 d has been used to explain the relatively high sensitivity to over-stimulation of NP-CTL (Zajac et al., 1998). However, prolonged presence of a stable protein, like NP, does not mean that CTL epitopes are generated continuously: It has been proposed by Yewdell and colleagues (Yewdell et al., 1996) that the majority of the MHC class I binding peptides are derived from defective ribosomal products (DRiPs), and thus heavily depend on protein neo-synthesis. Two studies supported this hypothesis (Reits et al., 2000; Schubert et al., 2000), and we showed recently for LCMV NP that no CTL epitopes were generated from this

long-lived protein, unless neo-synthesis took place (Khan et al., 2001). Therefore, the relevance of the observation that GP is not as persistent as NP in chronically infected mice to (over)stimulation of NP-CTL is not certain. We cannot formally exclude the possible contribution of cross-priming to the phenomenon we describe here. However, we don't expect cross-priming to be an important pathway for priming a CTL response against a pathogen that very efficiently infects dendritic cells.

We analysed the kinetics of presentation of NP- and of GP-derived epitopes by LCMV-WE and –Arm infected macrophages, because we argued that faster presentation of NP-derived epitopes could account for a better protective capacity on a per cell basis (Gallimore et al., 1998a), for better priming at limited viral load, and for higher sensitivity to exhaustion at high viral load. We found that, independent of the LCMV strain used, both NP-derived epitopes were sufficiently presented to stimulate CTL as early as 2.5 h after infection, whereas it took at least 8h before three different GP-derived epitopes were recognized. It is unlikely that different travelling times of particular MHC class I molecules from the endoplasmic reticulum to the cell surface account for the kinetic differences observed here, as H-2D^b is the restricting element of one NP-derived and of two GP-derived peptides. To formally exclude the possibility, that in vitro infected, thioglycollate-elicited macrophages differed with respect to antigen presentation from those cells that are naturally infected by LCMV in vivo, we used splenocytes isolated from LCMV-infected mice at different time points after infection as APC to stimulate GP- or NP-specific T cell hybridomas (Schwarz et al., 2000; Shastri and Gonzalez, 1993; Usherwood et al., 1999). Using this approach, we found similar kinetic differences between the presentation of GP- and NP-derived epitopes. We used above read outs for antigen presentation rather than peptide elution from MHC class I molecules, because the latter method requires a large amount of cells (>5x10^8 per time point and virus), that is impossible to obtain from other sources than from cell lines, the latter being probably abnormal in antigen processing or presentation.

As a result of faster kinetics of NP presentation, NP-CTL encounter their nominal peptide on every infected cell before GP-CTL do, and will therefore be activated earlier, and probably in the face of higher viral loads. The consequences of delayed GP presentation were demonstrated in vivo: pre-existing memory NP-CTL inhibited priming of GP-CTL, whereas pre-existing memory GP-CTL did not interfere with NP-CTL priming. In attempt to investigate consequences of kinetic differences in epitope presentation for in vivo priming in a direct manner, we analysed the expansion kinetics of NP- and GP-specific CTL at different, early time points after infection. To this end, we performed ICS at 3, 4, 5, 6, 7, 8, and 9 d after infection. We detected LCMV-specific CTL from d 5 onwards, however, we did not find substantial differences in expansion kinetics of NP- versus GP-CTL (data not shown). Detection of specific CTL by tetramers was unreliable as long as virus was still present (approximately until d 7), probably due to TCR downregulation. Kinetic differences of 8 h may not lead to detectable differences in expansion kinetics, because the time required for initial T cell activation and proliferation is much longer than 8 h, thus masking possible initial differences. Moreover, it may well be that possible differences in expansion kinetics could not be detected anymore by d 5, because most of the virus usually is eliminated by then, and a substantial part of the NP-CTL may be exhausted before they could expand to numbers that allow detection. No comparative data on naïve CTL_p frequencies are currently available. Alternatively, small differences in the frequencies of CTL_p could probably cover up differences in expansion kinetics.

Kinetic differences in epitope presentation may also explain the finding that an NP118-CTL escape mutant of LCMV, that still expressed the GP283 CTL epitope, was controlled slower than the wild type virus in BALB/c mice, but equally fast in CB6F1 mice (that could use an additional NP-derived epitope, NP396) (Weidt et al., 1998). It was found in the same study, that the 118-CTL escape mutant efficiently primed subdominant GP283-CTL in BALB/c mice, but not in CB6F1 mice, whereas LCMV-WE did not prime GP283-CTL in either mouse strain: Absence of an early NP-specific response in BALB/c

mice (but not in CB6F1 mice) results in slower virus control, and allows the priming of otherwise subdominant and later GP-specific responses. Results obtained by mathematical analysis of the LCMV-specific CTL response in BALB/c mice (De Boer et al., 2001) can also be explained by kinetic differences in epitope generation.

Based on our findings, we propose the general rule that CTL against early epitopes normally clear most of the virus, and are at risk to be exhausted if the initial viral load is relatively high. Experiments showing that "early" NP-CTL are 100 times more effective in protecting mice against LCMV compared with "late" GP-CTL ((Gallimore et al., 1998a), and van den Broek and Probst, unpublished data) fit this hypothesis. Data on specific CTL responses in other viral infections than LCMV seem to support the general applicability of our hypothesis. Vaccination of macaques with early Simian Immunodeficiency Virus (SIV)-proteins (Rev, Tat) induced a protective immunity that was substantially more effective than vaccination with late SIV-proteins (Pol, Gag) (Geretti and Osterhaus, 2001). Along the same line, longitudinal analysis of the Human Immunodeficiency Virus (HIV)-specific CTL response in individuals with a high HIV load revealed that Rev- and Tat-specific CTL were the first to disappear (Addo et al., 2001; Novitsky et al., 2001; van Baalen et al., 1997). In analogy to what we described here for LCMV infection, namely evidence for early activation of specific CTL followed by their gradual functional or even physical disappearance after they were unable to eliminate the antigen in due time, can be found in a variety of infections, such as HIV (Appay et al., 2000; Kostense et al., 2001), Hepatitis B (Lechner et al., 2000a; Lechner et al., 2000b; Sing et al., 2001) and Hepatitis C (Erickson et al., 2001), and also in tumor-bearing patients (Lee et al., 1999).

We propose that CTL against early viral proteins swiftly interfere with virus replication, resulting in efficient protection. If these "early" CTL fail in immediate virus control, they are activated in the face of higher viral load compared with "late" CTL and are therefore prone to be exhausted.

Thus, the observed absence of CTL specific for “early” determinants in persistent infections might not be the cause, but rather the consequence of viral persistence.

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Results Part II: Induction of Peripheral Tolerance of Cytotoxic T Cells

Inducible Transgenic Mice Reveal Resting Dendritic Cells as Potent Inducers of $CD8^+$ T Cell Tolerance

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Summary

Dendritic cells (DC) are inducers of immune responses par excellence. They also seem responsible for the induction of peripheral T cell tolerance. To investigate these opposite functions of DC, we generated a Cre/loxP-based system that allows inducible antigen presentation by DC in vivo. This enabled us to study the immunological consequences of antigen presentation by resting versus mature DC without adoptively transferring DC and with physiological numbers of endogenous, naïve responder T cells. We found that presentation of LCMV-derived CTL epitopes by resting DC resulted in antigen-specific tolerance, which could not be broken by subsequent infection with LCMV. On the other hand, antigen presentation by activated DC primed endogenous CTL to expand and to develop protective effector function.

Introduction

The induction of an efficient and protective adaptive immune response depends on the interaction between naïve antigen-specific T lymphocytes and professional antigen presenting cells (APC). Because of their unique features, such as migratory capacity and expression of costimulatory molecules and major histocompatibility complex (MHC) molecules, dendritic cells (DC) are considered the prototype of professional APC. DC are present as sentinels in peripheral tissues, where they can capture antigens that may be processed and presented to $CD4^+$ and $CD8^+$ T cells. Most DC in peripheral tissues have a resting phenotype: They can efficiently take up antigens, but do not present these productively to naïve T cells (Banchereau and Steinman, 1998b; Inaba et al., 1993). After interaction with products of microbial or viral pathogens (LPS, dsRNA, CpG DNA) through one or more members of the family of Toll-like receptors (TLR) (Kaisho and Akira, 2001), with proinflammatory cytokines (TNFα, IL-1β) or after ligation of surface CD40 (Bennett et al., 1998; Schoenberger et al., 1998), DC acquire an activated phenotype. Activated DC change their expression pattern of homing receptors, allowing efficient migration into T cells zones of lymph nodes (Roake et al., 1995; Sallusto et al., 1998b), and upregulation of costimulatory molecules, such as CD86 as

well as MHC class I and II molecules (Banchereau and Steinman, 1998b; Cella et al., 1997b), all contributing to an efficient priming of naïve T cells. There is ample evidence that antigen-presentation by activated DC is required and sufficient for the induction of T cell responses in vivo. Conditional depletion of $CD11c^+$ DC in vivo resulted in complete absence of CTL priming after infection with *Listeria monocytogenes* or with *Plasmodium yoelii* (Jung et al., 2002), identifying DC as the only APC being able to prime naïve CTL.

Bone marrow-derived antigen-bearing DC matured in vitro have been shown to induce specific T cell responses upon adoptive transfer (Banchereau and Steinman, 1998b; Bennett et al., 1998; Dhodapkar et al., 1999; Diehl et al., 1999; Inaba et al., 1993; Schoenberger et al., 1998; Schuurhuis et al., 2000b; Sotomayor et al., 1999). Evidence for induction of immunity as the major function of DC is mostly based on experiments that involved DC purification or culture in vitro, which are known to dramatically change the phenotype and immunostimulatory capacities of DC (Gallucci et al., 1999; Pierre et al., 1997b). Thus, the effect on naïve T cells of antigen presentation by DC in non-inflammatory, steady state situations was difficult to study using adoptively transferred DC. Nevertheless, it was found that adoptively transferred, freshly isolated and antigen-pulsed resting human DC induced silencing or inhibition of antigen-specific $CD8^+$ T cell effector functions in a limited number of healthy human recipients (Dhodapkar and Steinman, 2002a; Dhodapkar et al., 2001). In vivo targeting of antigen to $DEC205^+$ DC resulted in abortive proliferation of adoptively transferred TCR transgenic $CD4^+$ T cells (Hawiger et al., 2001b) and of $CD8^+$ T cells (Bonifaz et al., 2002b). In addition, autoantigen presented by $CD11c^+CD8\alpha^-CD4^+$ DC has been shown to mediate suppression of autoimmunity in vivo (Legge et al., 2002). Using adoptively transferred TCR transgenic T cells as a read out, antigen presentation of tissue-specific antigens by bone marrow-derived APC, that were identified as $CD11c^+CD8\alpha^+$ DC, was demonstrated under steady state conditions in draining lymph nodes. This induced abortive activation of the responding T cells, resulting in tolerance (Belz et al., 2002a; Hernandez et al., 2001a; Kreuwel et al., 2002; Kurts et al., 1997b; Liu et al., 2002a;

Scheinecker et al., 2002a). In contrast, in vivo targeting of SIINFEKL to $CD11c^+CD11b^+CD8\alpha^-$ DC was found to prime naïve, endogenous CTL (Guermonprez et al., 2002a). These conflicting data may be due to the use of different experimental systems, such as the analysis of transferred versus endogenous T cells, the frequency of naïve responder cells, or to different means of targeting of the antigen to DC *in vivo*, thereby "touching" one of their surface molecules.

Most experiments addressing the issue of tolerance induction by resting DC relied on adoptively transferred, TCR transgenic T cells as a read out, which results in abnormally high precursor frequencies. This is particularly disturbing as it has been shown that $CD8^+$ T cells can activate DC (Ruedl et al., 1999) or provide their own help (Wang et al., 2001a), given their frequency is sufficiently high. More important, the outcome of antigen presentation on naïve DC was found to vary dramatically with the number of TCR transgenic T cells transferred. Adoptive transfer of low numbers of TCR transgenic T cells specific for ovalbumin (OVA) or for influenza virus-derived heamagglutininin (HA) into mice expressing OVA or HA under control of the rat insulin promoter (RIP) resulted in initial activation of the TCR transgenic T cells, followed by complete tolerance induction. However, tolerance induction was less efficient at higher frequencies of specific T cells (Kurts et al., 1997b; Morgan et al., 1999a), and if precursor frequencies were even further increased, tolerisation of autoagressive T cells reverted to their activation, resulting in autoimmunity (Kurts et al., 1997b; Morgan et al., 1996a).

To circumvent problems mentioned above, we developed an approach that allows direct in vivo comparison of resting DC and activated DC for their respective capacity to prime, to be ignored by or to tolerize naïve $CD8^+$ T cells without the need for adoptive transfers of dendritic cells or of responding T cells, or other manipulations that might disturb the steady state situation in an immunocompetent mouse. Our system uses inducible expression and presentation of lymphocytic choriomeningitis virus (LCMV)-derived CTL epitopes by resting DC or by activated DC in vivo. We are able to induce a small percentage of all $CD11c^+$ DC to present transgenic CTL epitopes in

thymus, lymph nodes and spleen. We observed that activated, antigen-presenting DC efficiently primed naïve, endogenous CTL to proliferate and to differentiate into protective effectors, whereas resting, antigen-presenting DC induced antigen-specific tolerance, that could not be broken by a subsequent infection with LCMV.

Results and Discussion

Generation and characterization of CD11cCreER$_T$, ST33.396 and (CD11cCreER$_T$ X ST33.396) (=DIETER) mice

DIETER mice are double transgenic offspring of (CD11cCreER$_T$ X ST33.396) breeding, and express inducible Cre recombinase in CD11c$^+$ cells as a transgene. Cre activity is induced by a single i.p. injection of 2 mg tamoxifen (TAM), which results in the presentation of transgenic CTL epitopes by DC (Figure 1). Injection of DIETER mice with TAM together with an agonistic anti-CD40 antibody leads to presentation of transgenic CTL by activated DC, administration of TAM alone leads to antigen presentation by resting DC.

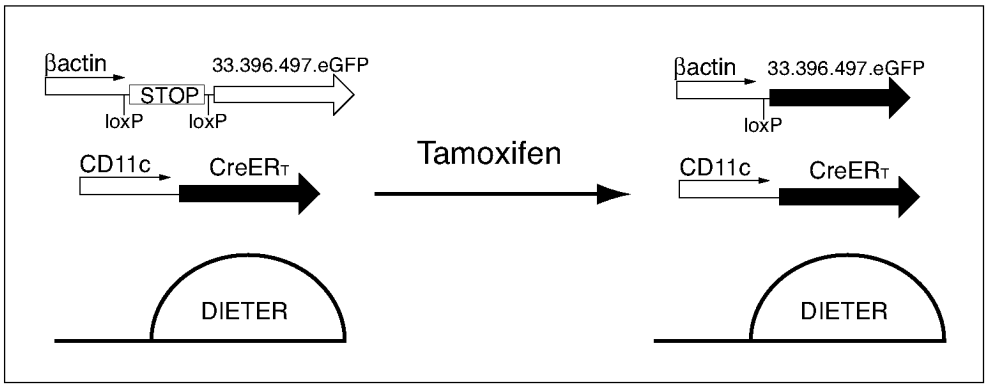

Figure 1: Generation of DIETER (ST33.396 X CD11cCreER$_T$) transgenic mice

DIETER mice are double transgenic offspring of CD11cCreER$_T$ X ST33.396 mice and therefore carry two transgenic constructs: (1) Cre recombinase as a fusion protein with the mutated hormone-binding domain of the human estrogen receptor (ER) under control of the DC-specific CD11c promoter. In absence of ER ligand, Cre recombinase is associated with heat shock proteins, which inhibit translocation of Cre into the nucleus. After association of ER with (synthetic) ligand (Tamoxifen), Cre can translocate into the nucleus and remove loxP-flanked nucleotides. (2) A transgene encoding LCMV-derived CTL epitopes (GP33-41/D^b and NP396-404/D^b) and an E. coli β-galactosidase-derived CTL epitope (βGal497-505/K^b) as a fusion protein with enhanced green fluorescent protein (eGFP). The transgene is separated from its ubiquitous chicken β-actin promoter by a loxP-flanked STOP cassette, which prevents expression of the transgene. Thus, DIETER mice only express the transgene, resulting in the presentation of transgenic CTL epitopes by CD11c$^+$ cells (DC), after administration of Tamoxifen.

Because we were unable to detect green fluorescence in $CD11c^+$ cells of DIETER mice after deletion of the STOP cassette, probably due to instability of the fusion protein, we bred $CD11cCreER_T$ mice with eGFP-based Cre-indicator mice (RAGE%) (Constien et al., 2001) and injected the F1 mice with TAM. We detected Cre activity as measured by green fluorescence exclusively in $CD11c^+$ cells (Figure 2A). Next, we isolated $CD11c^+$ cells from spleens of (RAGE% X $CD11cCreER_T$) F1 40 h after administration of 2 mg TAM and stained these with CD8α-APC/CD4-PE. We found Cre activity in all major subsets of splenic DC upon TAM injection (Figure 2B). After injection of 2 mg TAM, 4-8% of $CD11c^+$ in spleen, lymph nodes and thymus were found to express Cre (Figure 2C). No green fluorescence was detected in non-$CD11c^+$ cells (Figure 2A) or in $CD11c^+$ cells without TAM injection (Figure 2C). The percentage of $CD11c^+$ cells presenting the transgene is in a physiological range, as around 8% of splenic $CD11c^+$ stain positive for LCMV-NP at 2 days after infection with LCMV.

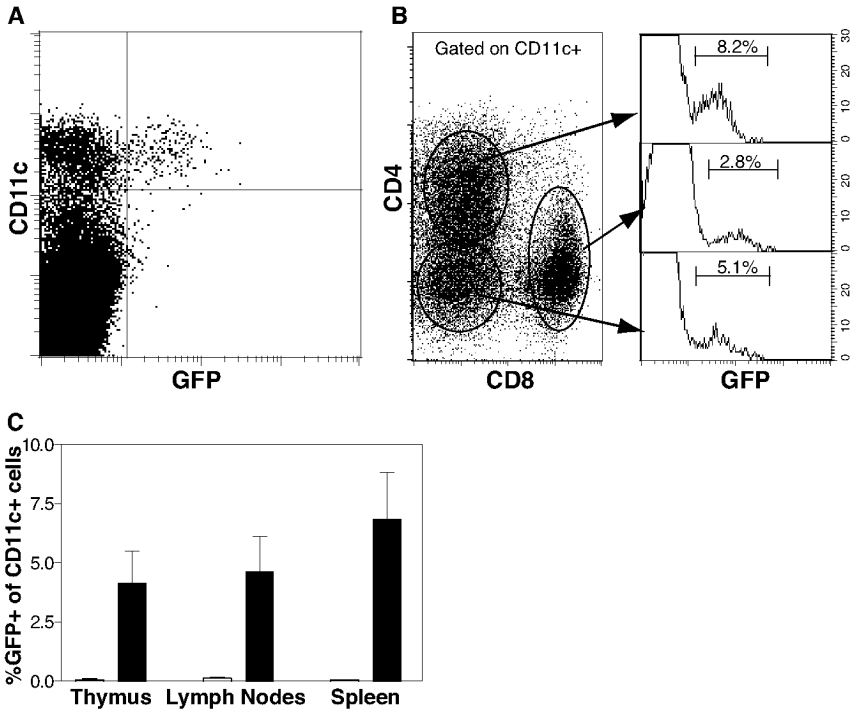

Figure 2: Characterization of CD11cCreER$_T$ mice

(A) Using RAGE% mice as indicator, Cre activity can be visualized by green fluorescencent protein expression. (CD11cCreER$_T$ X RAGE%)F1 mice were injected i.p. with 2 mg TAM, and 40 h later splenocytes were stained with anti-CD11c-PE to identify DC. **(B)** CD11c$^+$ cells were MACS-purified from spleen 40 h after administration of 2 mg TAM. CD11c$^+$ cells were stained with anti-CD4-PE and anti-CD8α-APC. Green fluorescence was detected in all subsets of DC. This experiment is one out of three comparable experiments. **(C)** Quantification of the number of CD11c$^+$ cells with Cre activity. (CD11cCreER$_T$ X RAGE%)F1 mice were injected i.p. with 2 mg TAM (solid bars) or solvent (open bars), and cells from thymus, mesenteric lymph nodes and spleen were stained for CD11c-PE 40 h later. The percentage of CD11c$^+$ cells with Cre activity is shown as the mean ± SD of three mice.

Antigen presentation by activated DC in DIETER mice primes endogenous, naïve CD8$^+$ cells

DIETER mice were injected with 2 mg TAM (to induce Cre activity resulting in presentation of GP33, NP396 and βGal497 by CD11c$^+$ cells in vivo), with 30 μg anti-CD40 (to activate DC in vivo), with 2 mg TAM plus 30 μg anti-CD40, or were left untreated. Staining of splenocytes for CD11c/CD86, CD11c/I-A^b and CD11c/CD54 at different time points after injection of 30 μg anti-CD40 followed by FACS analysis showed that all three markers, which are

characteristic for activated DC, were 3-6 fold upregulated with a maximum at 24-36 h after injection of anti-CD40 (data not shown), thus confirming previously published data (Hawiger et al., 2001b). The number of GP33-, NP396- and βGal497-specific $CD8^+$ T cells was quantified 7 d after the injections by tetramer staining of blood leukocytes. Injection of DIETER mice with TAM+anti-CD40 primed naïve, endogenous $CD8^+$ cells to expand substantially: around 15% of the $CD8^+$ cells were found to be GP33-specific, 1% were NP396-specific and 7% were βGal497-specific (Figure 3A). Naïve endogenous CTL did not expand to detectable levels after injection of DIETER mice with TAM or anti-CD40 alone (Figure 3A) or after injection of C57BL/6 mice with any of the combinations mentioned above (data not shown). No expansion was found in control groups at d 9 (data not shown). To test for antiviral protective effector function, the same mice were challenged with LCMV and viral titres were determined in spleen 4 days thereafter. High LCMV titres were found in nontransgenic C57BL/6 mice as well as in those DIETER mice that were injected with TAM, with anti-CD40, or that were untreated. No virus was found in DIETER mice previously injected with TAM+anti-CD40 (Figure 3B). This shows that only activated, antigen presenting DC prime naïve, endogenous CTL to expand (Figure 3A) and to acquire effector functions in vivo (Figure 3B). To exclude the contribution of a direct effect of anti-CD40 on $CD8^+$ T cells (Bourgeois et al., 2002), we injected DIETER mice with 2 mg TAM i.p. and activated DC with 250 _g poly-IC i.v. Like after injection of 2 mg TAM + 30 _g anti-CD40, we found priming of endogenous, transgene-specific $CD8^+$ T cells (data not shown). Moreover, adoptively transferred, mature bone marrow-derived DC isolated from (ST33.396 X Deleter) mice efficiently primed GP33-, NP396-, and βGal497-specific, endogenous CTL in C57BL/6 mice (data not shown) without the need for anti-CD40 injection. Deleter mice express Cre recombinase in a ubiquitous and constitutive fashion.

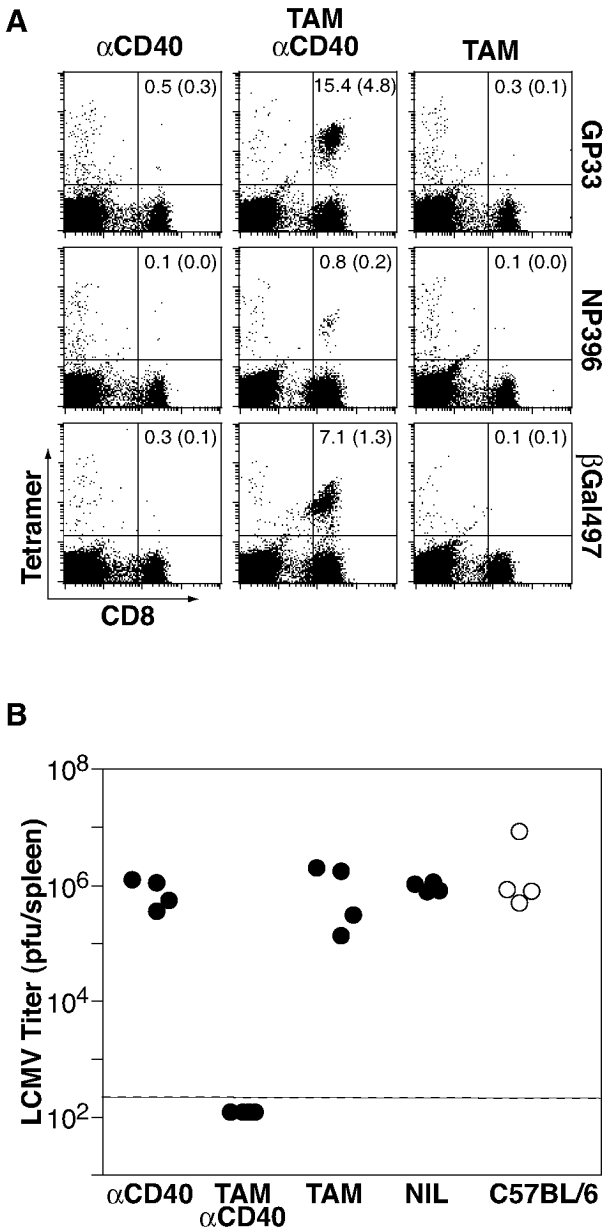

Figure 3: Antigen presentation by activated DC induces antigen-specific priming for expansion and for protective immunity of endogenous CD8⁺ cells

DIETER mice were injected on d -8 with 30 μg anti-CD40 i.v. (to activate DC), with 2 mg TAM i.p. (to induce presentation of CTL epitopes by DC) or with 30 μg anti-CD40 i.v. + 2 mg TAM i.p. **(A)** On d -1, blood leukocytes were stained with tetramers to detect expansion of transgene-specific CTL. Staining from one representative mouse out of four is shown. Values in the upper right quadrant represent the mean (+/- SD) percentage of tetramer⁺ cells within CD8⁺ cells. **(B)** Mice were challenged on day 0 with 100 pfu LCMV-Armstrong and viral titers were determined in spleens at d 4 using a focus-forming assay. One representative experiment of 2 is shown, values represent the titers of individual mice. The broken line represents the detection limit of the assay.

Antigen presentation by resting DC in DIETER mice induces peripheral, antigen-specific tolerance

To analyse whether antigen presentation by resting DC resulted in ignorance or in peripheral tolerance, we injected DIETER mice with 2 mg TAM. This treatment did not result in detectable priming of naïve CTL as determined by tetramer stains on d 5 (not shown) and on d 7 (Figure 3A). As control, we injected DIETER mice with solvent. On d 7, mice were infected with 100 pfu LCMV-Arm. Priming by LCMV was analysed 12 d after infection by tetramer staining, by intracellular staining for interferonγ (ICS) and by a CTL assay after restimulation with the relevant peptide in vitro. LCMV infection of C57BL/6 (H-2^b) mice induces CTL specific for three immunodominant epitopes: GP33, GP276 and NP396, two of which (GP33, NP396) are presented by DIETER DC after TAM injection. We found greatly reduced GP33- and NP396-specific CTL in TAM-treated, LCMV infected DIETER mice compared with solvent-treated mice (Figure 4A), but a normal GP276 response. The difference between TAM- and solvent-treated DIETER mice was even more pronounced in ICS (Figure 4B). This shows that antigen presentation by resting DC resulted tolerance of specific $CD8^+$ T cells, which could not be broken by subsequent infection with LCMV. Importantly, we found that LCMV infection primed GP276-specific CTL in TAM-treated DIETER mice to a degree comparable to that found in the three control groups (solvent-treated DIETER in Figure 4B; C57BL/6 not shown). Stimulation of splenocytes by PMA/Ionomycin resulted in similar percentages of IFNγ-producing $CD8^+$ T cells in TAM-treated and in control DIETER mice, suggesting that the total number of LCMV-specific CTL primed by infection was similar: CTL specific for dominant and subdominant epitopes other than GP33 and NP396 may compensate for the tolerized CTL populations. Priming of small numbers of GP33- or NP396-specific CTL or partial priming by resting DC could be excluded, because in vitro restimulation with relevant peptides did not result in substantial CTL activity (Figure 3C). The fact that GP276-CTL were efficiently primed and that the total number of LCMV-specific CTL was normal clearly shows that tolerance induced by antigen presenting resting DC

was an antigen specific phenomenon and was not due to transgenic artefacts resulting in defective antigen presenting cells.

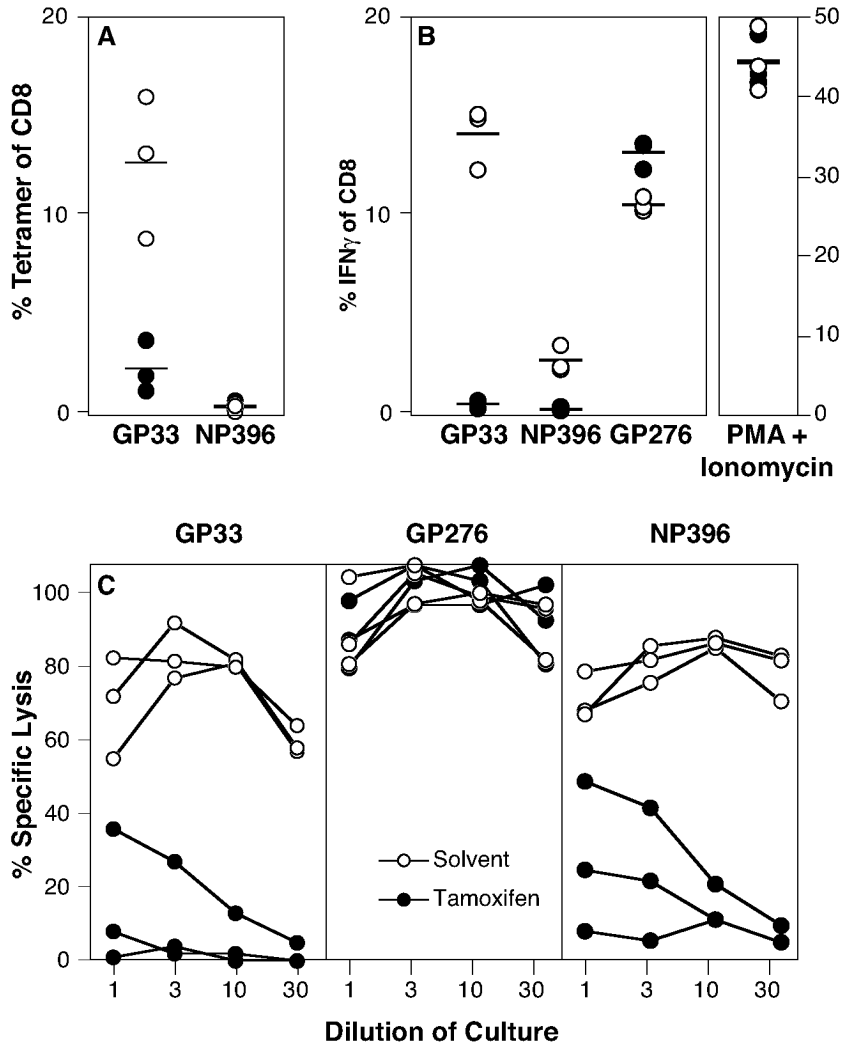

Figure 4: Antigen presentation by resting DC induces antigen-specific tolerance of endogenous CD8⁺ cells

DIETER mice were injected i.p. with 2 mg TAM (closed symbols) or with solvent (open symbols) on d –7. They were infected i.v. with 10^2 pfu LCMV-Armstrong on d 0, and the LCMV-induced CTL response was measured in the spleen on d 12 by tetramer staining **(A)**, by ICS **(B)** and by a cytotoxicity assay after restimulation in vitro **(C)**. Lysis of EL-4 targets without peptide was <15% of the specific lysis. Spontaneous lysis of targets was <15%. One representative experiment out of four is shown. Symbols or curves represent the values of individual mice.

DIETER DC did not present helper epitopes upon induction with TAM; therefore, we cannot conclude from our data whether additional and simultaneous presentation of helper epitopes by resting DC would affect the

outcome of $CD8^+$ T cell tolerance. However, based on the findings of Hawiger et al. (Hawiger et al., 2001b), we expect also that $CD4^+$ T cells would be tolerized and that thus $CD8^+$ T cell tolerance would be unaffected. Our data show that tolerance induced by resting DC is robust, as it could not be broken by a subsequent infection with LCMV, which is known to very efficiently prime CTL responses. It has been shown in other systems (Morgan et al., 1999a; Scheinecker et al., 2002a) that tolerance induced by resting DC that continuously crosspresent (neo)self-antigens is more limited in nature compared with tolerance in DIETER mice. The completeness of tolerance seemed to depend on the ratio between the number of autoreactive precursors and the number of tolerizing DC (Morgan et al., 1999a). We think that DIETER mice display such a robust tolerance, because the relatively high number of tolerizing DC (4%–8% of all $CD11c^+$ cells, Figure 2) compared with the limited number of endogenous T cell precursors (Blattman et al., 2002). In addition, direct presentation of intracellular antigen, as is the case in DIETER mice, is presumably more efficient than crosspresentation (Morgan et al., 1999a; Scheinecker et al., 2002a).

It has been described that, besides in DC, the CD11c promoter is also active in a certain percentage of activated T cells and intraepithelial lymphocytes (Huleatt and Lefrancois, 1995; Jung et al., 2002). To exclude that $CD8^+$ T cell tolerance described by us was due to antigen presentation by $CD8^+$ T cells followed by “fratricide”, rather than to antigen presented by resting DC, we performed the following control experiment. DIETER (Thy1.2) + C57BL/6.PL (Thy1.1) -> C57BL/6 (Thy1.2) bone marrow chimeras were injected with 2 mg TAM or with solvent on d 0 and were challenged with 100 pfu LCMV-Armstrong on d 7. The CTL response was analysed on d 12 after LCMV challenge (see also Figure 4). We found a strong LCMV-specific CTL response against GP33, GP276 and NP396 in solvent-treated, mixed chimeras in both the $Thy1.1^+$ (Figure 5) and the $Thy1.2^+$ (not shown) $CD8^+$ cells as measured by tetramer (Figure 5A) and by ICS (Figure 5B). More important, we found that the response against the two epitopes present in DIETER mice (GP33, NP396) was equally depressed in the Thy1.1 (Figure 5)

and the Thy1.2 (not shown) population in TAM-treated, mixed chimeras, whereas the GP276-specific CTL response was induced normally. If depressed responses in TAM-treated DIETER mice would have resulted from $CD8^+$-mediated fratricide, GP33- and NP396-specific responses should have been absent from the $Thy1.2^+$ population only. The fact that these responses were found to be absent from transgenic (Thy1.2, not shown) as well as from nontransgenic (Thy1.1) populations clearly shows that the phenomenon we observed is genuine T cell tolerance mediated by antigen presentation by resting DC in vivo.

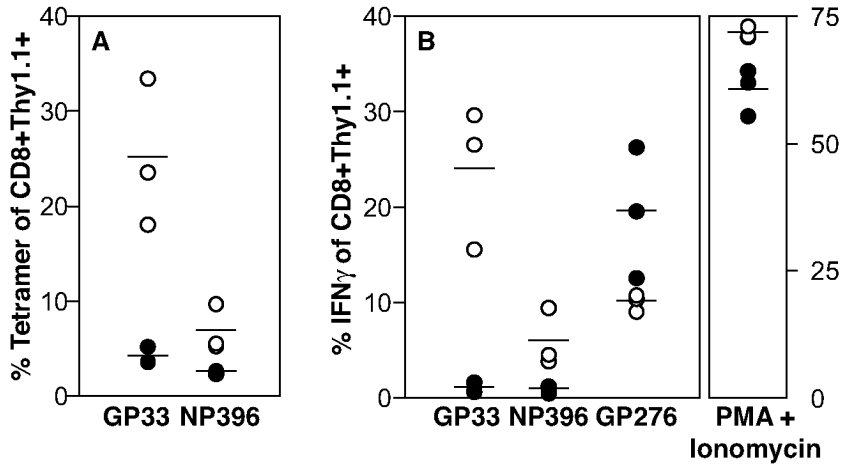

Figure 5: Tolerance is not due to promiscuous CD11c promoter activity resulting in fratricide

DIETER (Thy1.2) + C57BL/6.PL (Thy1.1) -> C57BL/6 (Thy1.2) bone marrow chimeras were treated with 2 mg TAM or with solvent on d 0, were infected with 100 pfu LCMV-Armstrong on d 7, and the LCMV-specific CTL response was analysed on d 18 by tetramer staining (A) and by ICS (B). Each group contained 3 mice.

We did not find proliferation of endogenous naïve CTL precursors as a result of antigen presentation by resting DC. This is in contrast with data published by others (Belz et al., 2002a; Bonifaz et al., 2002b; Hawiger et al., 2001b; Hernandez et al., 2001a; Kreuwel et al., 2002; Kurts et al., 1997b; Liu et al., 2002a; Scheinecker et al., 2002a), who always used adoptively transferred TCR transgenic T cells as responders. This discrepancy may be explained by differences in responding precursor frequencies: up to 10^6 per mouse after adoptive transfer of TCR transgenic cells, and about 100 per mouse (Blattman et al., 2002) in our experiments. We decided not to use adoptive transfers, to

assure that the steady state situation was not disturbed and, more importantly, to be able to study endogenous, polyclonal T cells. It is the consequence of this approach, that we can't conclude from our data whether or to which extent tolerance induction of T cells present at physiological precursor frequencies was preceded by abortive T cell activation or proliferation. At present, it is unclear by which mechanism immature DC induce peripheral tolerance, nor is it clear whether only naive or also effector or memory T cells can be tolerized by immature DC. It has been shown that interaction of T cells with antigen-presenting, immature DC results in abortive proliferation followed by deletion of the T cells (Hawiger et al., 2001b; Kurts et al., 1997b). However, other studies demonstrated that immature (Dhodapkar and Steinman, 2002a; Dhodapkar et al., 2001; Hugues et al., 2002) or partially mature (King et al., 2001; Menges et al., 2002) DC induce regulatory T cells that suppress T cell function by IL-10 (Dhodapkar and Steinman, 2002a; Dhodapkar et al., 2001; Legge et al., 2002) or by yet unidentified factors.

Our study proves, without adoptive transfers of DC, without targeting of antigens to DC and with physiological precursor frequencies of endogenous, naïve responder T cells, that DC mediate two completely opposite functions in the immune response, one being the induction of functional immunity and the other the induction of peripheral tolerance. The immunological outcome was found to depend solely on the activation status of the antigen presenting DC. Using a novel and versatile system that allows inducible expression of CTL epitopes on a cell type of choice, we showed that antigen presented by a small percentage of all $CD11c^+$ DC in activated condition was sufficient to prime naïve, endogenous CTL to expand and to develop effector functions, and that the very same DC in an resting status induced robust peripheral $CD8^+$ T cell tolerance. We showed that this peripheral tolerance is antigen-specific, is not due to abnormalities in the DC compartment, and can't be broken by a strong stimulus, such as infection with a replicating virus, or restimulation in vitro.

Materials and Methods

Generation of DIETER transgenic mice

DIETER mice are double transgenic offspring of a crossing of ST33.396 transgenic mice with CD11cCreER$_T$ transgenic mice. CD11cCreER$_T$ mice express Cre recombinase as a fusion protein with the mutated hormone-binding domain of the human estrogen receptor under control of the murine CD11c promoter (Brocker et al., 1997; Feil et al., 1996). In absence of ligand, Cre recombinase is associated with heat shock proteins, which inhibit translocation into the nucleus. After injection of ligand (Tamoxifen), Cre can translocate into the nucleus and remove loxP-flanked nucleotides. CD11cCreER$_T$ mice were generated by injecting the 8.6 kb NotI-SalI fragment of pCD11cCreER$_T$ into the pronuclei of fertilized C57BL/6 oocytes. pCD11cCreER$_T$ was constructed by cloning the 2 kb EcoRI fragment of pCreER$_T$ (Feil et al., 1996) into the EcoRI site of the plasmid CD11c.pDOI-5 (Brocker et al., 1997).

ST33.396 mice express a fusion protein of three CTL epitopes and eGFP under control of the ubiquitous chicken β-actin promoter (Ludin et al., 1996). To allow inducible expression of the transgene, a loxP-flanked STOP cassette (Sauer, 1993) was cloned between the promoter and the transgene. ST33.396 mice were generated by injecting the 6.5 kb NdeI-KpnI fragment from pβ-actinST33.396 into fertilized C57BL/6 oocytes. To generate pβ-actinST33.396, a 276 bp PCR fragment encoding aa 1-80 of the LCMV glycoprotein, which include the immunodominant, H-2D^b restricted CTL epitope GP33-41 (van der Most et al., 1996), was cloned into the EcoRI site of the vector peGFP-N2 (Clontech laboratories, Palo Alto, CA). Sequences encoding two further CTL epitopes, the H-2D^b restricted, LCMV nucleoprotein-derived NP396-404 (van der Most et al., 1996) and the H-2K^b restricted, β-galactosidase-derived βGal497-504 (Oukka et al., 1996) were introduced as synthetic oligonucleotides (396.497.1 and 396.497.2) into the SpeI/ApaI-digested construct between the LCMV-GP fragment and eGFP, in frame with both. To facilitate processing of transgenic CTL epitopes, they were flanked

by one aa from their original context. The resulting construct, pCMV3313.396.497eGFP encodes a fusion protein of viral and bacterial sequences with eGFP. To allow inducible expression of this fusion protein, a LoxP-flanked STOP cassette (Sauer, 1993) was cloned as a 1.6 kb blunted NotI fragment into the blunted HindIII site of pCMV3313.396.497eGFP, located in the multiple cloning site before the coding sequences. The resulting plasmid, pCMVSTOPL3313.396.497eGFP was digested with NotI, blunted, digested with NheI and the 2.7.kb fragment containing the protein coding sequences and the loxP-flanked STOP cassette was introduced into the MamI/SpeI-digested plasmid pβactin16 (Ludin et al., 1996).

Oligonucleotides

396.497.1:

5'TCGAATCTTTCAGCCACAGAATGGGCAGTTCATTCACTCTAGAATTATT TGCCCCATGTACGCCCGCGTGGATGGGCC3'

396.497.2:

5'CATCCACGCGGGCGTACATGGGGCAAATAATTCTAGAGTGAATGAACT GCCCATTCTGTGGCTGAAAGAT3'

Oligonucleotides were purchased PAGE-purified from Microsynth (9436 Balgach, Switzerland).

Mice

C57BL/6 and C57BL/6.PL (Thy1.1) were obtained from the Institut für Labortierkunde, University of Zürich. C57BL/6.PL mice were originally obtained from The Jackson Laboratories (Bar Harbor, Maine 04609, USA). CD11cCreER$_T$ mice express DC-specific, inducible Cre recombinase as a transgene (see above), ST33.396 mice carry a transgene encoding LCMV-derived CTL epitopes (GP33-41/D^b and NP396-404/D^b) and a β-galactosidase-derived CTL epitope (βGal497-505/K^b) as a fusion protein with enhanced green fluorescent protein (eGFP). The transgene is separated from its ubiquitous promoter by a loxP-flanked STOP cassette (see above). DIETER mice are double transgenic offspring of (CD11cCreER$_T$ X ST33.396) mice. RAGE% mice are eGFP-based Cre-indicator mice (Constien et al., 2001). All mice, except for RAGE%, had a pure C57BL/6 background. To

increase the number of available double transgenic (DIETER) mice, we generated DIETER -> C57BL/6 bone marrow chimeras by i.v. injection of DIETER bone marrow into lethally irradiated (9.5 Gy from a ^{57}Co-source), age- and sex-matched C57BL/6 mice. Alternatively, lethally irradiated C57BL/6 mice were grafted with a 1:1 mixture of bone marrow from age- and sex-matched DIETER and C57BL/6.PL mice. Bone marrow chimeras were given Borgal (Hoechst Roussel Vet, Lyssach, Switzerland) in the drinking water (1 mg/ml Sulfadoxin, 0.2 mg/ml Trimethoprim) during the first 2 weeks after reconstitution, and left at least for 8 weeks before use in experiments. Initial experiments showed that DIETER bone marrow chimeras and DIETER mice were indistinguishable in the outcome of all types of experiments described. Most experiments shown here were performed with DIETER bone marrow chimeras.

All animal experiments were performed in Switzerland or in Greece according to respective national laws on animal protection.

Virus

LCMV-Armstrong (ARM) was obtained from Dr, M. Oldstone (Scripps Clinic and Research Foundation, La Jolla, CA, USA) (Buchmeier et al., 1980). LCMV was propagated on L929 cells at a low multiplicity of infection (m.o.i.).

Cell Lines

EL-4 cells are dimethylbenzanthrene-induced thymoma cells of C57BL/6 origin. MC57G are methylcholanthrene-induced fibrosarcoma cells of C57BL/6 origin.

Induction of Cre recombinase activity and activation of DC in vivo

Cre activity was induced in vivo by injecting DIETER or (CD11cCreER$_T$ X RAGE%)F1 mice i.p. with 0.1 ml containing 2 mg of tamoxifen (TAM, ICN Biomedicals Inc., Aurora, Ohio, USA). TAM was suspended in 96% ethanol, 9 volumes of olive oil were added and TAM was dissolved at 37°C. Injection of 2 mg TAM into C57BL/6 mice did not interfere with the induction of antiviral CTL after LCMV infection, nor did it have any adverse effects on the mice (data not shown).

DC were activated in vivo by i.v. injection of 30 μg agonistic anti-CD40 antibody (FGK45.5, (Rolink et al., 1996). If mice were given both TAM and anti-CD40, this was done at the same time.

FACS Analysis

1) Induction and quantification of Cre activity in different subsets of DC

Double positive offspring from CD11cCreER$_T$ X RAGE% mice were injected i.p. with 2 mg TAM and 40 h later, CD11c$^+$ cells were isolated from thymus, spleen and mesenteric lymph nodes by collagenase/DNase digestion followed by magnetic separation using anti-CD11c-labeled MACS-beads according to instructions of the supplier (Miltenyi Biotec GmbH, Germany). CD11c$^+$ cells were stained anti-CD4-PE and anti-CD8α-APC (all PharMingen). Dead cells were excluded by propidium iodide. Green fluorescence was used as a read out for Cre activity (Constien et al., 2001). We found green fluorescence in CD11c$^+$ cells as early as 15 h after injection with TAM, and at least until 75 h after TAM injection. The number of CD11c$^+$GFP$^+$ cells in spleen, lymph nodes and thymus peaked around 40 h (data not shown).

2) Staining with tetrameric MHC class I-peptide complexes

Tetrameric complexes containing biotinylated H-2D^b or -K^b, β_2-microglobulin, the relevant peptide and extravidin-PE were generated, and staining was performed as described (Altman et al., 1996; Probst et al., 2002).

3) Intracellular Staining for Interferon-γ (ICS)

Splenocytes were incubated for 6 h at 37°C with 10^{-6}M of the specific peptide, with medium alone or with PMA+ionomycin in the presence of 5 μg/ml Brefeldin A. Cells were surface stained with anti-CD8α-PE (PharMingen) stained intracellularly with anti-mouse-IFNγ-FITC (clone AN18, PharMingen).

Cytotoxicity Assay

Splenocytes were restimulated in vitro for 5 days in the presence of 50U/ml recombinant mouse IL-2 with irradiated, thioglycollate-elicited peritoneal macrophages, that were loaded with 10^{-8} M of the relevant peptide. Threefold dilutions of the cultures were tested for cytotoxic activity using ^{51}Cr-labeled EL-4 cells that were or were not loaded with 10^{-6} M of the relevant peptides as targets in a 5 h chromium release assay. The percentage of specific lysis was

calculated as follows: % specific release = (experimental release – spontaneous release) ÷ (maximal release – spontaneous release) X 100%.

Protection Assay

Mice were infected i.v. with 100 pfu LCMV-Armstrong, spleens were removed 4 d later and were homogenized. Tenfold serial dilutions of spleen suspension were used to infect monolayers of MC57G fibroblasts for 48 h. LCMV was detected using the LCMV-NP specific antibody VL-4 as described (Battegay et al., 1991b).

Acknowledgments

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General Discussion

Organisms are confronted with numerous pathogens, which spend at least a part of their life cycle inside the host cell. All pathogens have a characteristic tissue tropism resulting in the infection of certain cells of the host. Considering all possible pathogens, each of with its own tropism, one has to assume that most body cells can be infected. For an efficient defence against intracellular pathogens it is therefore crucial that the surveillance by cytotoxic CD8+ T cells covers all compartments of the body. This is possible because almost all host cells are able to present antigens derived from intracellular pathogens on their surface in the context of host MHC class I molecules. Once a specific CD8+ T cell has been activated by its specific antigen and has acquired full effector function, it will be able to detect infection in all types of cells and fight these infections by elimination of the infected cell. While every possible cell that presents the specific peptide/MHC class I complex can be eliminated by activated effector CTL, only few cells meet the requirements necessary for the induction or priming of naïve CD8+ T cells. The induction of CD8+ T cell responses depends on a coordinated interaction of naïve CD8+ T cells and professional antigen presenting cells (APC) that present the specific antigen associated with MHC class I molecules. Despite decades of intensive research on the induction of CTL responses, numerous questions regarding the anatomical sites where priming can take place, the type of APC that can activate naïve CD8+ T cells, the costimulatory interactions (signal 2) that are mandatory in addition to the TCR:peptide/MHC class I interaction, the impact of innate defence, and the factors that determine to which possible antigenic determinants the CD8+ T cell response is focussed, remain unanswered.

Antigen presenting cells

Lafferty and co-workers, who noted that the rejection of histoincompatible organ grafts was dependent on donor leukocytes trapped in the graft, first demonstrated the requirement for specialized stimulator cells for T cell activation in a series of classical experiments (Lafferty et al., 1975; Talmage

et al., 1976). Based on their ability to express MHC class II and co-stimulatory molecules and to take up antigens, B cells, macrophages and DC are thought to have the capability to stimulate naïve T cells and are thus referred to as professional antigen presenting cells (APC). During the past twenty years however, a large body of evidence has accumulated that supports the suggestion that DC are the only cells fulfilling all criteria required to be a professional APC. In fact, DC were found to be unchallenged in their potency to prime allogeneic T cell responses both *in vivo* and *in vitro* (Lechler and Batchelor, 1982; Steinman et al., 1983). In addition, only DC and not B cells nor macrophages are found in the T cell area of secondary lymphoid organs where the naïve T cells are located. Furthermore, using confocal microscopy of sectioned lymph nodes following local infection with Vaccinia Virus, it was shown that, although both DC and macrophages were infected with Vaccinia Virus, only DC productively presented antigen to naïve T cells (Norbury et al., 2002). In elegant experiments using B cell chimerical chickens, resting B cells were shown to be unable of stimulating naïve T cells (Lassila et al., 1988). Moreover, normal T cell responses to a wide variety of stimuli were found in B cell deficient mice, supporting the notion that B cells do not play a crucial role in the priming of naïve T cells *in vivo* (Epstein et al., 1995). Recently, a novel diphtheria toxin based system was developed, which allows the inducible ablation of DC *in vivo* (Jung et al., 2002). Depletion of the DC compartment resulted in a complete inability to prime CD8+ T cells responses against *Listeria monocytogenes* and *Plasmodium yoelii.* Taken together, it appears likely that of the three cell types that have been considered as professional APC, namely macrophages, B cells and DC, only CD11c+ DC are able to prime CTL responses *in vivo.*

Naïve CD8+ T cells circulate through secondary lymphoid organs. These tissues are located at strategic sites where pathogenic microbes may enter the body, either by penetrating the skin, crossing of a mucosal barriers, or by injection into the blood by arthropod vectors (Chaplin, 2003). The unique cellular composition and micro-architecture of the secondary lymphoid organs is thought to enhance the sensitivity of antigen recognition and to provide an

optimal environment for T cell activation (Fu and Chaplin, 1999). It has been suggested that the ability of an antigen to reach a secondary lymphoid organ is a key requirement for CTL priming (Zinkernagel et al., 1997). Although it has been demonstrated recently that priming of adoptively transferred, TCR transgenic CD8+ T cells also can occur in the bone marrow (Feuerer et al., 2003), mice lacking lymph nodes and Peyer's Patches due to a spontaneous genetic defect (*aly/aly* mice) failed to mount a protective CTL response against lymphocytic choriomeningitis virus (LCMV) (Karrer et al., 1997), thus demonstrating the crucial role of secondary lymphoid organs in the induction of immune responses.

DC reside in peripheral tissues, particularly at the interface with the environment such as the skin and mucosal surfaces. In these tissues they make up 1-2% of the total cell number. DC can efficiently take up antigens, including pathogens, in peripheral organs and can subsequently transport these into lymphoid organs (Banchereau and Steinman, 1998a; Guermonprez et al., 2002b). Thus, DC form the physical link between the peripheral tissues where pathogens usually invade and the secondary lymphoid organs where priming of naïve T cells can take place.

In the light of their unique capability to prime T cells, another crucial feature of DC immediately becomes apparent: In order to initiate T cell responses against different kinds of pathogens, DC have to take up these pathogens or pathogen-derived antigens and present their epitopes to T cells. Immature DC have been reported to phagocytose almost all kinds of bacteria as well as yeast cells such as *Candida albicans* and *Saccharomyces cerevisiae* and parasites such as *Leishmania major, L. Donovani* and *L. mexicana.* (Guermonprez et al., 2002b). Moreover, DC are equipped with a large number of endocytic receptors (Guermonprez et al., 2002b). C-type lectins bind to carbohydrate structures on the surface of pathogens or on pathogen-derived antigens in a calcium dependent manner using highly conserved carbohydrate recognition domains. (Figdor et al., 2002). Several transmembrane C-type lectins have been identified on the surface of DC, including the mannose receptor (MR, CD206), DEC205 (CD205), Langerin (CD207) and DC-SIGN

(CD 209). Another type of endocytic receptor, such as receptors for the Fc portion of immunoglobulins (FcR) and complement receptors (CR) mediates the uptake of opsonised antigens. Murine immature DC have been shown to express FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (Esposito-Farese et al., 1995; Fanger et al., 1996), and the complement receptors CR3 and CR4 (Reis e Sousa et al., 1993). Because antigens presented by MHC class II molecules are generally derived via the endocytic pathway (Germain and Margulies, 1993), it is obvious that DC can prime T helper responses against a large number of pathogens, which they have taken up by one of the mechanisms described above. In contrast, antigen presentation by MHC class I molecules is generally restricted to cytoplasmic antigens actively synthesized within the APC (Rock and Goldberg, 1999). Antigens derived from pathogens that infect and replicate in DC, such as LCMV (Sevilla et al., 2000), can immediately access the MHC class I antigen presentation pathway and can therefore readily induce CTL responses. However, many intracellular pathogens use receptors for cell entry that are not expressed by DC. CTL responses against such pathogens can be primed according to two theoretical possibilities: either DC have to be able to transport antigen that has been taken up from outside the cell into their MHC class I antigen presentation pathway, or DC need to have developed strategies to become infected by a large number of different pathogens. Early evidence for the first possibility, referred to as “cross presentation”, was obtained from experiments in which the immunization of mice with cells expressing minor histocompatibility antigens led to the induction of a CTL response restricted to MHC alleles of the host that were not present in the cells used for immunization. (Bevan, 1976a; Bevan, 1976b). Two pathways have been suggested for cross presentation. In the first, the antigen could be loaded on MHC class I either in the endocytic compartment, a process that should be independent of degradation by the proteasome and TAP transport, or alternatively in the ER in which case endocytosed antigen would have to be transported to the cytosol and subsequently into the ER in a TAP-dependent fashion. Indirect evidence has been reported for both pathways (reviewed in (Yewdell et al.,

1999)). It has been shown that DC have developed a membrane transport pathway linking the lumen of the endocytic compartments with the lumen of the cytosol (Rodriguez et al., 1999; Thery and Amigorena, 2001), and this pathway was shown to be restricted to DC. More recently it was demonstrated that this pathway of cross presentation involves fusion of phagosomes with the ER, allowing the phagosome to acquire the protein machinery necessary for export of the antigens to the cytosol as well as the entire MHC class I loading complex (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). Taken together, although the relevance of cross priming for the induction of CTL responses against pathogens is still a matter of intense debate (Melief, 2003; Zinkernagel, 2002), a mechanism is emerging how DC could use their enormous potential to phagocytose many different kinds of pathogens not only for the generation of T helper cell responses but also for CTL priming.

Recently, some evidence for a unique susceptibility of DC to infection by a broad spectrum of pathogens became available. It was found that the C-type lectin receptor DC-SIGN could be used by various pathogens to infect DC. Infection of DC via DC-SIGN was reported for Ebola virus (Alvarez et al., 2002), Human Cytomegalovirus (Halary et al., 2002) *M. tuberculosis* (Geijtenbeek et al., 2003; Tailleux et al., 2003) and Dengue virus (Tassaneetrithep et al., 2003). Moreover, it was demonstrated that Poliovirus, a virus that uses a receptor for cell entry that is absent in mice, could infect and replicate in mouse DC *in vivo*, resulting in priming of a CTL response (Freigang et al., 2003)

In order to efficiently stimulate naïve T cells, DC have to undergo a differentiation process, called maturation. This process involves a number of morphological changes, including loss of adhesive structures, cytoskeleton reorganization and the acquisition of high cellular motility (Winzler et al., 1997). At the same time DC undergo a switch in chemokine receptor expression that allows the cell to move through the lymph into the T cell zones of the secondary lymphoid tissue (Dieu et al., 1998; Sallusto et al., 1998a;

Yanagihara et al., 1998). The immunogenicity of the antigen presenting DC is dramatically enhanced during DC maturation by the increased formation of stable MHC-peptide complexes (Cella et al., 1997a; Pierre et al., 1997a; Turley et al., 2000), the upregulation of costimulatory molecules, such as CD86, CD80 and other B7 family members (Carreno and Collins, 2002; Inaba et al., 1994; Tseng et al., 2001), and the synthesis of cytokines that influence T cell proliferation and differentiation (Ebner et al., 2001; Granucci et al., 2003; Granucci et al., 2001; Langenkamp et al., 2003). Thus, the maturation status of antigen presenting DC appears to be the critical checkpoint for the initiation of T cell responses and the ability of an antigenic stimulus to induce DC maturation appears to be responsible for the immunogenicity of that antigen (Bonifaz et al., 2002a; Hawiger et al., 2001a; Probst et al., 2003a).

Activation of DC is generally seen in response to pathogens or signs of their presence (Reis e Sousa, 2001). Janeway proposed that the induction of adaptive immunity depends on a distinct, innate recognition event involving primitive receptors, termed pathogen recognition receptors (PRR), which bind conserved microbial structures, the so-called pathogen associated molecular patterns (PAMPs) (Janeway, 1992). Macrophages, DC, mast cells, neutrophils, eosinophils, and NK cells express different innate PRR. These cells can become activated during an inflammatory response, which usually accompanies an infection, and then rapidly differentiate into short-lived effector cells whose main role is to get rid of the infection. However, in certain cases the innate immune system is unable to deal with the infection, and activation of an adaptive immune response becomes necessary. In these cases, the innate immune system can alert the adaptive immune system about the nature of the pathogenic challenge.

A major class of PRRs consists of the Toll like receptors (TLRs). In mammals at least ten members of the TLR family have been described until now. TLRs specifically recognize microbial components, such as LPS, unmethylated CpG motifs, bacterial peptidoglycans, double stranded (ds) RNA naturally occurring during replication of some viruses, flagellin and many more (Takeda et al., 2003). However, not only cell surface PRRs, but also PRRs in the cytoplasm

have been described. For example protein kinase R has been shown to recognize dsRNA in the cytoplasm of infected DC and to induce DC maturation (Diebold et al., 2003). In addition to direct recognition of pathogens by receptors on the DC or inside the DC, indirect ways of DC activation by pathogens have also been described. Indeed DC maturation is induced in response to inflammatory mediators such as TNF-α, IL-1β, or PGE-2 that are secreted in response to infections (Banchereau et al., 2000). Furthermore, ongoing T-cell responses can activate DC via ligation of surface CD40 by CD40L, which is expressed on the surface of activated $CD4^+$ T cells (Caux et al., 1994; Schuurhuis et al., 2000a).

The upregulation of particular surface molecules, including CD54, CD80, CD86, MHC II and CCR7, is usually used as hallmark for DC activation. Interestingly, although most PAMPs or TLR signals induce DC activation according to the abovementioned criteria, these stimuli differ dramatically in their capacity to licence DC for T cell priming (Schwarz et al., 2003). This implies that upregulation of these particular surface molecules is not the most important feature that discriminates « priming » from « nonpriming » DC. Using our recently developed model of inducible antigen presentation by DC *in vivo*, we will address this issue by comparing the priming capacity of DC after activating them through particular PRR.

Matzinger extended the concept of Janeway by proposing that rather than recognizing pathogens directly, the immune system recognizes “danger” signals, originating from cell destruction associated with the infection. These danger signals were suggested to be molecules that are usually sequestered inside the cell, but are released upon cellular stress or necrotic cell death (Matzinger, 1994). Evidence for this model came from the observation that heat shock proteins, which are released upon necrotic, but not apoptotic, cell death, can activate DC (Basu et al., 2000; Singh-Jasuja et al., 2000) Furthermore, uric acid was recently identified as a danger signal that is released from injured cells (Shi et al., 2003).

Activation of CD8+ T cells

Recognition of the complex of antigenic peptide and the restricting MHC molecule by the T cell receptor (TCR) is the key event in the induction of T cell responses. Antigen recognition by the TCR is a process of remarkable sensitivity and specificity. T cells can respond to as few as 50-100 specific MHC/peptide complexes presented on the surface of an APC (Demotz et al., 1990; Harding and Unanue, 1990) and they can discriminate these complexes from a vast excess of complexes of irrelevant peptides bound to the same MHC molecule. Mass spectrometry analysis of peptides eluted from MHC class I molecules showed that as many as 1000-2000 different peptides were presented at greater than 10 copies by the product of a single MHC class I gene (Hunt et al., 1992). Given this high sensitivity and specificity, the affinities found for the interaction of a TCR with its specific MHC/peptide complex are surprisingly low (Karjalainen, 1994). Affinity constants are typically around 1-90 μM with half-lives of around 2-25s at 25°C (Davis et al., 1998). Two different models have been proposed to explain the exquisite specificity of T cell antigen recognition: occupancy models and kinetic models. Occupancy models suggest that the potency of a ligand to activate a T cell is dependent on its concentration and on its affinity for the TCR (Schodin et al., 1996; Sykulev et al., 1995). Accordingly, the signalling events within the cell are a function of the number of receptors occupied at a given time. Kinetic models suggest that the off rate of the TCR:MHC/peptide interaction determines whether T cell activation takes place. (McKeithan, 1995; Rabinowitz et al., 1996). Engagement of the TCR by its ligand only results in a productive activation signal if a series of phosphorylation steps is subsequently induced. This leads to a temporal lag between ligand binding and receptor signalling. Dissociation of the ligand prior to completion of the signalling process results in lack of T cell activation or even in a negative signal for this particular T cell (McKeithan, 1995; Rabinowitz et al., 1996).

A consensus for the apparent paradox between high antigen sensitivity of T cells and the low affinity of the TCR became apparent when it was reported that one MHC/peptide complex could serially trigger a large number of TCRs

(Valitutti et al., 1995). It was found that ~100 MHC/peptide complexes could induce the downregulation of up to 1800 TCRs. This downregulation was a specific consequence of TCR triggering, as the expression of irrelevant TCRs was unaffected in cells with dual TCRs (Valitutti et al., 1995). Thus, through serial triggering only a few agonists can trigger the number of TCR required for activation (Valitutti and Lanzavecchia, 1997). This hypothesis was supported by the finding that not only mutations that shortened the half-life of the TCR:MHC/peptide interaction, but also those which led to very stable TCR:MHC/peptide interactions blocked T cell activation, presumably by limiting the ability of an MHC/peptide complex to serially engage many TCRs. (Hudrisier et al., 1998; Kalergis et al., 2001).

Based on the finding that even ligands that bind the TCR with a rather short half-life, can induce TCR activation provided that the period of stimulation is long enough, it was suggested that certain intermediates in the TCR signalling cascade are relatively stable and enable the T cell to "count" the serial triggering events (Rosette et al., 2001). The time that is required to activate a naïve T cell is therefore dependent on antigen concentration as well as on TCR binding kinetics. In experiments where naive TCR transgenic T cells were stimulated *in vitro*, 20 hr of sustained stimulation were found to be necessary for full T cell activation, and this was decreased in the presence of costimulation (Iezzi et al., 1998). When large numbers of relevant peptide/MHC molecules were presented by mature DC, the required stimulation time was even further reduced to 6 hr ((Lanzavecchia et al., 1999) Costimulation is therefore thought to play an important role in determining the extent and rate of accumulation of signalling intermediates. In a culture system using fibroblasts that were stably transfected to express antigen and the costimulatory molecule B7.1 (CD80) even 2 hr of stimulation were sufficient for T cell activation (van Stipdonk et al., 2001). However, if the T cells were adoptively transferred into naive recipient mice after activation *in vitro*, sustained proliferation *in vivo* was only found if they had been activated for at least 20 hr (van Stipdonk et al., 2003) (Kaech and Ahmed, 2001). A

central limitation of these studies is that they examined the kinetics of T cell activation in static systems *in vitro* using co-cultures of T cells and APC or even plate bound TCR ligands. In an attempt to mimic T cell activation in a tissue environment, Gunzer *et al.* incorporated T cells and DC presenting the specific antigen in a three-dimensional collagen matrix (Gunzer et al., 2000). The interactions between T cells and APC, as measured by time-lapse video microscopy were rather short-lived and highly dynamic, which is in contrast with results found in conventional liquid cultures. Moreover, a single T cell was found to make sequential contacts with one or multiple APC resulting in an estimated interaction time of 2-6 hr over a period of 3 days. However, it was impossible for technical reasons to deduce a minimal interaction time for T cell activation in these experiments. Several recent studies have attempted to directly visualize priming of naïve T cells within the intact lymph node. In a study that used confocal microscopy, it was found that T cells stably interacted with antigen-presenting DC for at least 15 hr before they actually became activated (Stoll et al., 2002). Other studies used two-photon microscopy to visualise T cell priming in deeper areas of the lymph node. Whereas one of these studies found both stable clusters and "swarming" of T cells around a centre that was probably occupied by an antigen presenting cell (Miller et al., 2002), the other study reported stable interactions of T cells and antigen-presenting DC for the entire tracking time (11 min on average), which suggests a stable DC-T cell contact in the range of hours rather than minutes (Bousso and Robey, 2003). A major limitation in these studies is that the lymph nodes had to be removed from the animal for observation, thus making it difficult to ensure physiologic conditions, such as oxygen pressure, inside the organ (von Andrian, 2002). Recently, two-photon microscopy of surgically exposed lymph nodes of anaesthetized mice has been reported, and the use of this technique to visualise T cell priming may lead to a better understanding of the interactions between T cells and APC *in vivo* (Miller et al., 2003).

The estimated time that naïve T cells and APC should interact to allow for priming varies depending on the experimental system used. Nevertheless, based on published data it seems reasonable to assume that the required interaction time is in the order of hours, rather than minutes or days. One consequence might be that kinetic differences in epitope generation have an impact on the actual size of the CTL response. During an infection, one and the same APC usually will present epitopes derived from different proteins of the pathogen. Inherent to pathogen replication, some proteins will be expressed before others, which is likely to result in kinetic differences in the presentation of individual epitopes. We have used LCMV infection as a model system to show that rather small differences in the kinetics of protein expression have a dramatic effect on the T cell response against antigens derived from these proteins (Probst et al., 2003b). We found that epitopes derived from LCMV nucleoprotein (NP) were detectable by CD8+ T cells 6-10 hours before those derived from LCMV glycoprotein (GP). Our finding that priming of GP-specific T cells was impossible in the presence of NP-specific CTL - but not vice versa- can be interpreted such that 6-10 hours of antigen presentation is sufficient for the activation of CD8+ T cells *in vivo*.

In order to successfully fight an infection, not only must CD8+ T cells be activated quickly and undergo massive clonal expansion but they must also develop effector functions, such as the secretion of IFNγ and cytolytic activity. Two studies proposed a link between the number of divisions a cell underwent and the effector function it acquired (Gudmundsdottir et al., 1999; Oehen and Brduscha-Riem, 1998). Several studies have suggested that upon activation, T cells enter a default program of proliferation and differentiation without the need for further activating signals (Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001). However, some observations in the past years have led to the notion that under certain circumstances, proliferation and the gain of effector function may be uncoupled (Hernandez et al., 2002). TCR transgenic CD8+ T cells were found to undergo abortive proliferation and deletion upon adoptive transfer into mice expressing their cognate antigen in

the pancreas (Morgan et al., 1999c). This was converted into T cell activation and subsequent diabetes if activated, antigen-specific CD4+ T cells were transferred at the same time. Surprisingly, activation of APC with an agonistic anti-CD40 antibody could not substitute for T help in this system as although injection of anti-CD40 resulted in strong expansion of the transferred CD8+ T cells, they failed to develop effector functions. The ability of the transferred CD8+ T cells to secrete IFNγ was induced if the mice were given IL-12 at the day after adoptive transfer (Hernandez et al., 2002). Using a novel transgenic mouse model, we found that antigen presentation by dendritic cells led to the generation of an antiviral protective CD8+T cell response if the DC were activated by CD40 ligation (Probst et al., 2003a). Although these CD8+ T cells protected the mice against a viral challenge infection, they displayed only weak effector functions if tested *in vitro* for their capacity to produce IFNγ or to lyse target cells. (Probst and van den Broek, unpublished results). Interestingly, we only found detectable *in vitro* effector function if the time of *in vivo* antigen presentation was increased and if we additionally injected mice with recombinant IL-12. The observed discrepancy between protective capacity in a challenge infection and the lack of *in vitro* detectable effector function might simply reflect the fact that the challenge infection itself can induce IL-12 expression, which results in full T cell activation. Our observation is in line with two recently published studies suggesting a requirement of antigen persistence and IL-12 (Curtsinger et al., 2003a) or innate immune stimulation (Storni et al., 2003) for the development of effector function in CD8+ T cells. The experiments discussed above are in concordance with the hypothesis that a combination of antigen (signal 1) and costimulation (signal 2) is not sufficient for acquisition of full effector function by CD8+ T cells, but that a third signal is required. This signal can be delivered by IL-12 (Curtsinger et al., 2003a; Curtsinger et al., 2003b; Curtsinger et al., 1999), which is produced by DC and macrophages upon encounter of microbial stimuli (Gately et al., 1998) (Curtsinger et al., 2003a), but not after activation via CD40 ligation alone (Schulz et al., 2000). It is not known at present whether other inflammatory mediators can play the role of signal 3 as well.

It has been pointed out above that the size of a T cell response is largely governed by the amount of presented antigen and by the duration of antigen presentation. Together with the frequency of specific CTL precursors that is present in the naïve host, these parameters determine to which epitopes of a pathogen the CTL response will be focussed (Chen et al., 2000; Yewdell and Bennink, 1999). Extent and duration of the presentation of a particular determinant depends on the efficiency of the various steps of antigen processing and on the expression level of the protein from where the antigenic peptide is derived.

We have shown that in addition to the amount, the kinetics of protein expression, which is inherent to the life cycle of the pathogen, also influences the efficiency of T cell priming (Probst et al., 2003b). Subdominance of an epitope is usually thought to be the result of suboptimal T cell stimulation. Whereas this might be correct for infections that induce weaker CTL responses, the situation appears to be different in infections where CTL responses are efficiently induced. Our study using infections with LCMV strains of different virulence has revealed that subdominance of the CTL response against certain epitopes was not caused by limited priming, but rather resulted from partial exhaustion of those specificities that received the strongest stimulation (Probst et al., 2003b). Exhaustion of CTL was first described as a form of high zone tolerance upon infection of mice with high doses of rapidly spreading LCMV isolates (Moskophidis et al., 1993). Under these circumstances, all LCMV-specific T cells disappeared and the virus persisted for the lifetime of the animal. The notion that exhaustion may not be complete and can differently affect T cells of different specificities was derived from LCMV infections of immunocompromised mice (Ou et al., 2001) or from studies using LCMV isolates that lead to a more chronic type of infection (Zajac et al., 1998). These persistent virus isolates induced different stages of functional impairment in T cells of different specificities, depending on the level of cognate antigen presentation. IL-2 production and cytotoxic activity were the first effector functions to be compromised, followed by TNFα

production and finally by IFNγ production. The ultimate step in this hierarchical loss of different effector functions was physical deletion (Wherry et al., 2003). Our observation that partial exhaustion of particular specificities already occurs in situations in which the virus is readily controlled clearly demonstrates that exhaustion is not restricted to chronic infections but can occur whenever excessively high levels of antigen presentation are reached. The fact that some specificities can already undergo partial exhaustion whereas others are still unaffected supports the original suggestion that exhaustion is a result of the complete induction of all available precursors followed by terminal differentiation and activation induced cell death (Moskophidis et al., 1993; Zinkernagel et al., 1993) rather than being a result of the elimination of professional antigen presenting cells (Borrow et al., 1995; Sevilla et al., 2000).

Tolerance of CD8+ T cells

The T cell receptor is generated through a series of combinatorial events including the somatic rearrangement of germ line V, D and J genes and the association of α and β chains and of random processes such as the deletion and insertion of non germ line encoded amino acid residues at the junctions of the germ line gene segments. While these processes ensure a receptor repertoire of enormous diversity that allows the recognition of a wide spectrum of pathogen encoded peptides, they may also generate T cells that recognize peptides of self proteins. As many of the effector functions of activated T cells can lead to extensive tissue destruction it is vital for the organism to prevent the activation of these autoreactive T cells. The immune system has developed complementary safety mechanisms that ensure T cell tolerance against self proteins (Stockinger, 1999). The first and probably most important of these safety mechanisms is the induction of central tolerance, which is mediated by negative selection during T cell maturation in the thymus. In the process of negative selection all immature thymocytes that receive a strong TCR signal undergo apoptosis (Kappler et al., 1987). Thus, negative selection eliminates all T cells specific for antigens presented within the thymus. This

includes ubiquitously expressed antigens as well as antigens expressed specifically by cells of the thymus and antigens that are present in high concentrations in the serum. As thymic dendritic cells are efficient APC for the induction of negative selection (Matzinger and Guerder, 1989), central tolerance also covers all proteins that are specifically expressed by DC, thus eliminating the risk of priming against DC encoded proteins when DC are activated during an infection. However, the expression of many proteins is exclusively restricted to certain peripheral tissues. These proteins may never reach the thymus and therefore it was difficult to envisage how autoreactive T cells against these tissue specific antigens could be eliminated by negative selection. However, recent data suggested that central tolerance might cover a much wider range of antigens than previously assumed. Medullar thymic epithelial cells (mTECs), which are the second type of antigen presenting cells that has been shown to efficiently induce negative selection (Burkly et al., 1993), were shown to have the unique capacity to express various tissue specific antigens (Derbinski et al., 2001). Analysis at the protein level revealed that each of the analysed tissue specific antigens was expressed only by a small proportion (1-5%) of mTECs. This suggests that random derepression of genes encoding tissue specific antigens occurs in mTECs and this enables the mTECs to induce negative selection of T cells specific for these antigens (Kyewski et al., 2002). Further support came from the identification of the gene defect in the autosomal recessive multiorgan autoimmune disease APECED. Patients with this disease were found to express a defective form of the transcription factor AIRE (Bjorses et al., 1998). AIRE deficient mice, which also developed a multiorgan autoimmune disease, showed a marked reduction in ectopic expression of tissue specific antigens by thymic epithelial cells (Anderson et al., 2002). Furthermore, it could be demonstrated in a transgenic mouse model that AIRE-driven expression of a tissue specific antigen in the thymus indeed led to thymic deletion of the T cells specific for this antigen (Liston et al., 2003). Promiscuous gene expression by mTECs therefore appears to provide a mechanism by which central tolerance can be efficiently established for antigens that are otherwise expressed in a tissue

specific manner. However, despite its striking efficiency, central tolerance appears to be incomplete. Self reactive T cells can escape negative selection and enter the periphery (Bouneaud et al., 2000) (Slifka et al., 2003). The fact that these autoreactive T cells usually do not become activated by self antigen in the periphery suggests the existence of other safety mechanisms that act downstream of central tolerance induction.

As previously discussed, it is known that dendritic cell maturation is necessary for T cell activation. Presentation of self antigen in the periphery will therefore fail to activate self specific T cells in the absence of stimulation by microbial compounds or innate immune reactions. However, the immune system is able to mount pathogen specific responses during the course of an infection without activating autoreactive T cellsat the same time, even though the same DC often presents both self antigens and pathogen-derived antigens. Two major mechanisms have been suggested to prevent the occurrence of autoimmunity in this situation. First, the self antigen could simply stay outside the lymphoid organs and is therefore ignored by the T cells (Zinkernagel, 1996) or second, self antigen that reaches the lymphoid organs in the absence of an infection or inflammation induces peripheral T cell tolerance (Miller et al., 1998).

Interestingly, evidence for both concepts was obtained in very similar experimental systems using transgenic mice that express an antigen under control of a tissue specific promoter. Two studies used transgenic mice that express the glycoprotein or nucleoprotein of LCMV under control of the rat insulin promoter, which drives expression specifically in pancreatic β-islet cells (RIP-GP, RIP-NP mice) (Ohashi et al., 1991) (Oldstone et al., 1991). In these models, expression of the viral protein in the pancreas did not lead to spontaneous diabetes. This was not due to the induction of tolerance because T cells specific for the neo self antigen could be activated by an LCMV infection. These activated T cells infiltrated the islets and mediated islet cell destruction resulting in diabetes. It was concluded from these models, that antigens expressed in peripheral tissues are ignored by naïve self specific T

cells unless these T cells are activated by an infection. Upon activation these T cells can then recognize their antigen in peripheral tissues and mediate autoimmune tissue destruction.

A completely different outcome was observed in a model where another viral glycoprotein, the hemagglutinin of influenza virus, was expressed in β-islet cells (Ins-HA mice). Similar to the mice expressing LCMV proteins in the pancreas, these mice did not develop spontaneous diabetes. However, Ins-HA mice showed markedly reduced CD4+ and CD8+ T cell responses against HA upon infection with influenza virus (Lo et al., 1992), which is in striking contrast to the LCMV situation. Moreover, Ins-HA mice did not show T cell infiltrates in the islets and did not develop diabetes upon infection with influenza virus. This was due to T cell tolerance rather than to a lack of accessibility of the antigen as adoptive transfer of naive T cells from wild type mice prior to infection led to infiltration of the islets and to diabetes (Lo et al., 1992). Experiments using Ins-HA mice that were thymectomised and subsequently irradiated and reconstituted with wild type bone marrow and a neonatal wild type thymus graft, demonstrated that T cell tolerance in Ins-HA mice was induced in the periphery and not in the thymus (Lo et al., 1992). When TCR transgenic CD8+ T cells specific for a HA peptide were adoptively transferred into Ins-HA mice, they proliferated in the lymph node that drains the pancreas, but not in any other lymph node. This demonstrated that HA expressed in the pancreas reached the draining lymph node, where it was presented to T cells. This resulted in activation of HA-specific T cells followed by deletion (Morgan et al., 1999b), with T cell tolerance as a consequence. The time required for tolerization depended on the number of adoptively transferred HA-specific T cells and on the amount of antigen expressed by the β-cells. Homozygous Ins-HA mice, which express higher levels of HA in their β-cells than heterozygous Ins-HA mice, showed accelerated tolerance induction, indicating that the efficiency of peripheral tolerance induction by the cross presentation of a tissue specific antigen critically depends on the amount of antigen that reaches the secondary lymphoid tissue. (Morgan et al., 1999b). In transgenic mice expressing ovalbumin under control of the rat

insulin promoter (RIP-OVA mice), Kurts *et al.* were able to study the dose dependence of peripheral tolerance induction over an even wider range of antigen concentrations (Kurts et al., 1999). When they compared two transgenic mouse strains that expressed either a high (1ng/μg of islet protein) or a low (<0.03ng/μg of islet protein) amount of secreted ovalbumin in their pancreatic β cells, they found that only the high expressers were able to delete adoptively transferred naive ovalbumin specific transgenic OT-1 CD8+ T cells. OT-1 cells transferred into low expressers were not deleted but rather ignored the antigen and remained fully functional. The amount of ovalbumin expressed by the low expressers was sufficient to target the islet cells for destruction, because adoptive transfer of activated OT-1 cells resulted in diabetes in both high and low expresser RIP-OVA mice (Kurts et al., 1998). Based on these findings the authors suggested that peripheral tissue antigens fall into three categories. Firstly, self antigens expressed at high levels can access both the relatively efficient direct presentation pathway, targeting the cell that expresses the antigen for lysis by effector CTL, and the relatively inefficient cross presentation pathway on APC in the secondary lymphoid organs, leading to tolerization of naïve CD8+ T cells. Secondly, peripheral tissue antigens that are expressed at lower levels cannot access the cross presentation pathway but can sensitize peripheral tissue cells for recognition by activated CTL, and thirdly, antigens that are expressed at very low levels do not gain access to either pathway and thus can never be recognized by CTL.The finding that some neo-self antigens expressed by β-islet cells were ignored (Ohashi et al., 1991) (Oldstone et al., 1991) whereas others induced peripheral tolerance (Kurts et al., 1997c; Lo et al., 1992) may simply reflect the expression level of the respective neo-self antigen. However, the expression level may not be the only factor that determines whether a tissue specific antigen will be ignored or will induce tolerance. It has recently been documented that the LCMV-WE glycoprotein sequence used by Ohashi *et al.* for the generation of RIP-GP mice carries a point mutation that blocks processing and surface expression of the glycoprotein (Beyer et al., 2001). Thus, although this protein can efficiently access the MHC class I

presentation pathway of the cell that expresses it, it cannot be transported into the draining lymph node to induce tolerance. The consequences of this point mutation may explain the difference between the RIP-GP mouse published by Ohashi *et al.* and the RIP-GP or RIP–NP mice published by Oldstone *et al.*. The latter used cDNA from the Armstrong strain of LCMV for the transgenes encoding LCMV-GP and LCMV-NP. As these sequences do not carry mutations that prevent folding and processing of the proteins it can be expected that these transgenes drive the expression of stable proteins, although this has not been formally shown. Interestingly, these mice developed diabetes only late after LCMV infection (2-6 months) and showed significant reduction of reactivity to the LCMV antigen expressed in the islets when analysed at the peak of the LCMV-induced CTL response. This suggests that tolerance was indeed induced in most autoreactive CTL precursors. In later experiments by the same group using newly generated RIP-GP and RIP-NP mice partial tolerance and delayed onset of diabetes was assigned to thymic expression and central tolerance (von Herrath et al., 1994). However, thymic expression was not shown for the original set of transgenic mice (Oldstone et al., 1991). In summary, these results suggests that the stability and cellular localization of a protein are also critical for its ability to induce peripheral T cell tolerance. Stable and secreted proteins expressed at relatively high levels are thus likely to induce peripheral tolerance whereas intracellular proteins expressed at low levels and with a short half-life are likely to be ignored by naïve T cells.

Several recent reports have demonstrated that in the course of an infection T cells can be primed against co-administered, normally non-immunogenic proteins (Le Bon et al., 2003) (Brimnes et al., 2003). Thus, there is a risk for autoimmunity inherent to peripheral self antigens that can reach the secondary lymphoid organs. Peripheral tolerance, which is induced and maintained continuously by the presentation of self antigen in the secondary lymphoid organs, thus acts as a safety mechanism to prevent activation of self specific T cells and autoimmunity in the course of an infection. Ignorance might occur in those cases where the amount of self protein is very low or

where the protein has a limited half life. Both of these would reduce transport of the protein into secondary lymphoid organs to such an extent that priming, and thus the risk for autoimmunity, is limited.

Although the abovementioned experiments with transgenic mice expressing neo self antigens in the pancreas clearly indicated that proteins induce peripheral tolerance if they reach the lymphoid tissue in sufficient quantities, the nature of the APC involved remained undefined. Experiments with bone marrow chimeras demonstrated that bone marrow-derived APC are required for the induction of peripheral tolerance of both CD8+ Tcells (Kurts et al., 1996) and CD4+ Tcells (Adler et al., 1998). Recently, DC were identified as the only cell type presenting the antigen in the pancreatic lymph node of animals that express a neo self-antigen in the pancreas (Belz et al., 2002b; Kurts et al., 2001). This finding was confirmed in a non transgenic situation in a study focusing on the gastric proton pump H^+/K^+ ATPase whose expression is restricted to gastric parietal cells (Scheinecker et al., 2002b). Using a monoclonal antibody, the gastric H^+/K^+ ATPase was detected in a small percentage of the DC in the gastric draining lymph node and DC isolated from this lymph node were able to activate a CD8+ T cell clone specific for the H^+/K^+ ATPase. These results clearly demonstrate presentation of tissue specific self antigens by DC in the draining lymph node. As this occurs continuously, the effect on antigen specific T cells has been difficult to study without adoptively transferring antigen-specific T cells. Adoptive transfer, however, is known to disturb the steady state situation and to result in artificially high precursor frequencies. Depending on the number of self antigen specific T cells that were adoptively transferred, all possible outcomes varying from peripheral tolerance to autoimmunity were found as a result of self antigen presentation by steady state DC (Kurts et al., 1997a; Morgan et al., 1999b; Morgan et al., 1996b).

To overcome these limitations, we have generated a mouse model in which antigen presentation on steady state DC can be induced without further manipulations (Probst et al., 2003a). Our results clearly demonstrate that antigen presentation by steady state DC, as it usually occurs for tissue

specific self antigens, results in tolerance of specific CD8+ T cells. Of note, our inducible transgenic mice (DIETER mice) express the antigens only in a small fraction (approximately 5%) of their DC. This might mimic the physiological situation better than the recenty reported targeting of antigens to DC via a monoclonal antibody, which reached almost all DC (Bonifaz et al., 2002a; Hawiger et al., 2001a) Moreover, our data demonstrate that antigen presentation by a rather small number of steady state DC is sufficient for the induction of robust CD8+ T cell tolerance. It should be noted, however, that we cannot determine the amount of self-peptide:MHC complexes presented per induced DC in DIETER mice and that we thus cannot exclude that the MHC class I restricted presentation of an endogenously synthesized antigen in DIETER DC is more efficient than the cross presentation of exogenous, tissue specific antigens. As DIETER mice express the transgene on all major subsets of DC we can also not determine whether only a subset of DC is responsible for induction of peripheral tolerance, as has been suggested (Liu et al., 2002b; Scheinecker et al., 2002b). However, preliminary data using another line of DIETER mice, which express the transgene only in CD8α^+ DC, indicated this subset was sufficient for both tolerance induction in a steady state situation and priming in an activated (Probst and van den Broek, unpublished results).

The mechanisms underlying the induction of tolerance are still poorly defined. Both dominant mechanisms, such as the induction T cells with regulatory properties that in turn could inhibit the proliferation of effector T cells (Dhodapkar and Steinman, 2002b; Jonuleit et al., 2000; Mahnke et al., 2003) and recessive mechanisms, such as the deletion or silencing of specific cells (Bonifaz et al., 2002a; Hawiger et al., 2001a) have been suggested. When we infected DIETER mice with LCMV after induction of antigen presentation by resting DC we found that tolerance affected only those T cells specific for the two epitopes present in the transgene (Probst et al., 2003a) . The CTL response against other epitopes of LCMV was even enhanced when these two specificities had been tolerized, resulting in a total LCMV specific CTL response of the same size as in untreated animals. This clearly demonstrates

that peripheral tolerance induced by antigen presentation on resting DC is strictly antigen specific and does not involve a dominant suppressive mechanism, at least in our model where only CD8+ T cell epitopes are presented.

A central principle of our experimental approach is that we study tolerization of T cells that are present at physiological precursor frequencies thereby avoiding any disturbance of the physiological steady state. It is inherent to this approach that we are unable to detect the rare specific cells that are tolerized by antigen presentation on resting DC. We therefore cannot discriminate between silencing and deletion as mechanisms of tolerance induction. However, when we drastically increased the precursor frequency by adoptively transferring transgenic CD8+ T cells specific for the LCMC GP33 epitope, we found that GP33 presentation by resting DC resulted in proliferation of the transferred T cells, which was similar in extent to the proliferation induced by GP33 presentation by activated DC. However, in contrast to cells primed by activated DC, most of these T cells were unable to produce IFNγ after 6h of restimulation *in vivo* (Probst and van den Broek, unpublished observations). This is in concordance with observations made in other models where adoptive transfer of TCR transgenic CD8+ T cells was used to study tolerance induction (Bonifaz et al., 2002a; Hawiger et al., 2001a; Hernandez et al., 2001b; Kurts et al., 1997c). Given that the outcome of these experiments depended on the number of transferred T cells (Kurts et al., 1997a; Morgan et al., 1999b; Morgan et al., 1996b) and that CD8+ T cells, if present at high frequencies, can activate DC (Wang et al., 2001b), it is unclear whether results obtained with adoptively transferred responder T cells reflect the physiological situation or are a result of the artificially high precursor frequency. We are convinced that it is crucial to study the mechanisms of peripheral tolerance induction at physiological T cell frequencies.

Little is known about the molecular processes that lead to tolerance induction following antigen presentation by resting DC. It has been suggested, that tolerance is the default reaction for T cells receiving TCR stimulation in the

absence of sufficient costimulation or cytokine signalling and that it follows a pathway that involves signaling by the proapoptotic molecule Bim and which can be inhibited by the anti-apoptotic molecule Bcl-2 (Davey et al., 2002). Besides tolerance induction as a result of missing signals, there are indications of more active mechanisms that may be used by resting DC to tolerize T cells. Recent studies have implicated indeolamine 2,3-dioxygenase (IDO), the rate limiting enzyme of the tryptophan catabolism, in tolerance induction by DC (Mellor and Munn, 2003). IDO is expressed by some DC in humans (Munn et al., 2002) and mice (Fallarino et al., 2002). Induction of IDO has been shown to induce T cell apoptosis *in vitro* (Fallarino et al., 2002). It has been suggested that some intermediates of the tryptophan catabolism might have a proapoptotic effect on T cells (Grohmann et al., 2003). In addition, several members of the B7 family expressed by DC seem to play a role in negative regulation of T cell activity and maintenance of immunological tolerance (Carreno and Collins, 2002). The prototype of these receptor ligand pairs is the interaction of CTLA-4 expressed on T cells with B7.1 (CD80) or B7.2 (CD86) expressed on DC (Chambers et al., 2001). Mice deficient in CTLA-4 develop a fatal lymphoproliferative disorder (Waterhouse et al., 1995) that starts very early after birth. Other inhibitory receptors of this family are PD-1, which binds the B7 family members PD-L1 (B7-H1) and PD-L2 (B7-DC)(Dong et al., 1999; Latchman et al., 2001) and BTLA, which binds B7x (Watanabe et al., 2003). Deletion or blockade of these molecules has been shown to induce or exacerbate autoimmune diseases (Ansari et al., 2003; Nishimura et al., 1999). Although this suggests a role for these molecules in the maintenance of T cell tolerance, these pathways have never been investigated in models of peripheral tolerance induction. The inducible transgenic mice described in this thesis provide us with a well defined model for peripheral tolerance induction. Crosses of DIETER mice to mice deficient for molecules implicated in T cell tolerance, adoptive transfer of T cells that lack candidate molecules, or the use of monoclonal antibodies to block putative inhibitory interactions will allow us to investigate the molecular mechanisms of peripheral tolerance induction by resting DC.

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