Year: 2003

Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8+ T cell tolerance

Probst, Hans Christian; Lagnel, Jacques; Kollias, George; van den Broek, Maries

Abstract: 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 enables us to study the immunogical consequences of antigen presentation by resting versus mature DC without adoptively transferring DC and with physiological numbers of endogenous, naive 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.

DOI: https://doi.org/10.1016/S1074-7613(03)00120-1

Immunity, Vol. 18, 713–720, May, 2003, Copyright ©2003 by Cell Press

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

Hans Christian Probst,[1] Jacques Lagnel,[2] George Kollias,[2] and Maries van den Broek[1,*]
[1]Institute of Experimental Immunology
University of Zürich
CH-8091 Zürich
Switzerland
[2]Institute for Immunology
Biomedical Research Centre Alexander Fleming
16672-Vari
Greece

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 enables us to study the immunogical consequences of antigen presentation by resting versus mature DC without adoptively transferring DC and with physiological numbers of endogenous, naive 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 naive 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 naive T cells (Banchereau and Steinman, 1998; 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., 1998) and upregulation of costimulatory molecules, such as CD86 as well as MHC class I and II molecules (Banchereau and Steinman, 1998; Cella et al., 1997), all contributing to an efficient priming of naive 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 naive 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, 1998; Bennett et al., 1998; Dhodapkar et al., 1999; Diehl et al., 1999; Inaba et al., 1993; Schoenberger et al., 1998; Schuurhuis et al., 2000; 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., 1997). Thus, the effect on naive T cells of antigen presentation by DC in noninflammatory, 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, 2002; 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., 2001) and of CD8$^+$ T cells (Bonifaz et al., 2002). In addition, autoantigen presented by CD11c$^+$CD8α^-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$^+$CD8α^+ 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., 2002; Hernandez et al., 2001; Kreuwel et al., 2002; Kurts et al., 1997; Liu et al., 2002; Scheinecker et al., 2002). In contrast, in vivo targeting of SIINFEKL to CD11c$^+$CD11b$^+$CD8α^- DC was found to prime naive, endogenous CTL (Guermonprez et al., 2002). 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 naive responder T 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

*Correspondence: maries@pathol.unizh.ch

help (Wang et al., 2001), given that their frequency is sufficiently high. More important, the outcome of antigen presentation on naive 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., 1997; Morgan et al., 1999), and if precursor frequencies were even further increased, tolerization of autoagressive T cells reverted to their activation, resulting in autoimmunity (Kurts et al., 1997; Morgan et al., 1996).

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 naive $CD8^+$ 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 naive, 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.

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 hr 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).

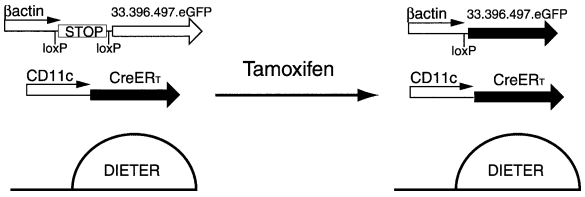

Figure 1. Generation of DIETER (ST33.396 X $CD11cCreER_T$) Transgenic Mice

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.

After injection of 2 mg TAM, 4%–8% of $CD11c^+$ in spleen, lymph nodes, and thymus were found to express Cre activity (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 $CD11c^+$ cells stain positive for LCMV-NP at 2 days after infection with LCMV (data not shown).

Antigen Presentation by Activated DC in DIETER Mice Primes Endogenous, Naive $CD8^+$ 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- to 6-fold upregulated with a maximum at 24–36 hr after injection of anti-CD40 (data not shown), thus confirming previously published data (Hawiger et al., 2001). The number of GP33-, NP396-, and βGal497-specific $CD8^+$ T cells was quantified 7 days after the injections by tetramer staining of blood leukocytes. Injection of DIETER mice with TAM+anti-CD40 primed naive, 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). Naive endogenous $CD8^+$ T cells 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

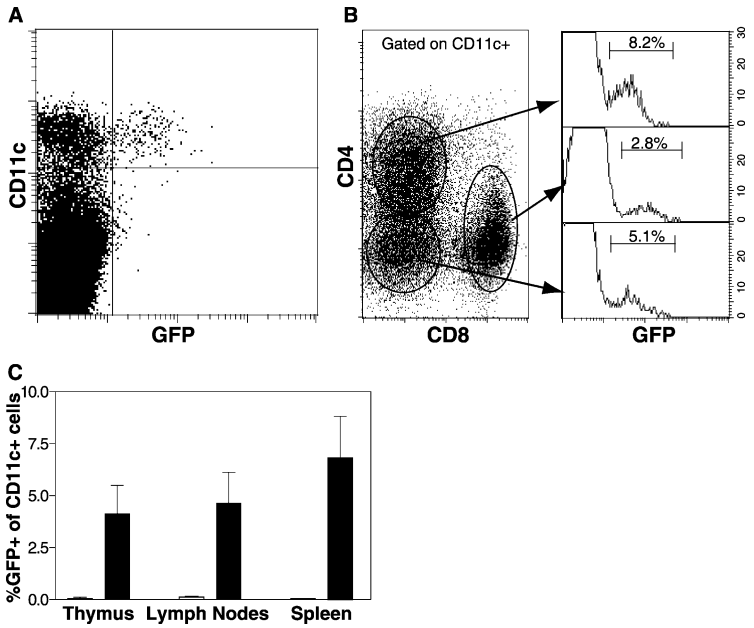

Figure 2. Characterization of CD11cCreER$_T$ Mice

(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 hr later splenocytes were stained with anti-CD11c-PE to identify DC.

(B) CD11c$^+$ cells were MACS-purified from spleen 40 hr 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 hr later. The percentage of CD11c$^+$ cells with Cre activity is shown as the mean $\pm$ SD of three mice.

for antiviral protective effector function, the same mice were challenged with LCMV and viral titers were determined in spleen 4 days thereafter. High LCMV titers were found in nontransgenic C57BL/6 mice as well as in those DIETER mice that were injected with TAM or 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 naive, 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 μg poly-IC i.v. Like after injection of 2 mg TAM + 30 μg 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.

Antigen Presentation by Resting DC in DIETER Mice Induces Peripheral, Antigen-Specific Tolerance

To analyze 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 naive CD8$^+$ T cells 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 analyzed 12 days 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 in 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; solvent- or TAM-treated 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 artifacts resulting in defective antigen-presenting cells. 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. (2001), we expect also that CD4$^+$ T cells would be tolerized and that thus CD8$^+$ T cell tolerance would be unaffected.

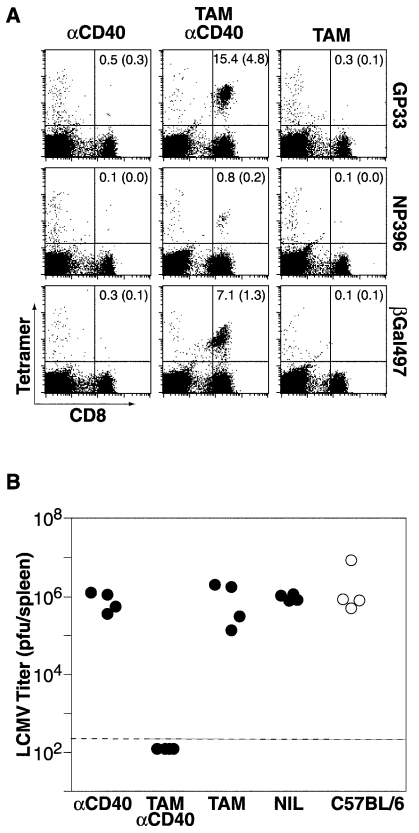

Figure 3. Antigen Presentation by Activated DC Induces Antigen-Specific Priming for Expansion and for Protective Immunity of Endogenous CD8$^+$ Cells

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 two is shown; values represent the titers of individual mice. The broken line represents the detection limit of the assay.

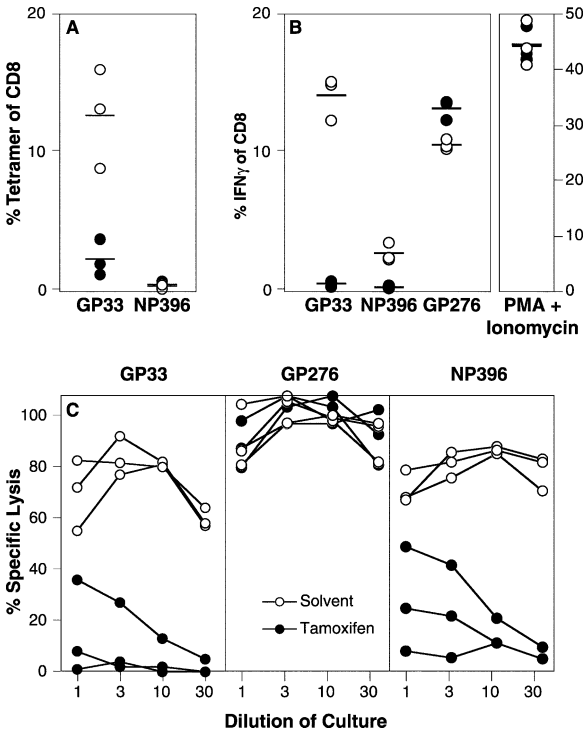

Figure 4. Antigen Presentation by Resting DC Induces Antigen-Specific Tolerance of Endogenous CD8$^+$ Cells

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.

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., 1999; Scheinecker et al., 2002) 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., 1999). 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., 1999; Scheinecker et al., 2002).

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 analyzed 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$^+$ (data 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) populations in TAM-treated, mixed chimeras, whereas the GP276-specific CTL response was induced

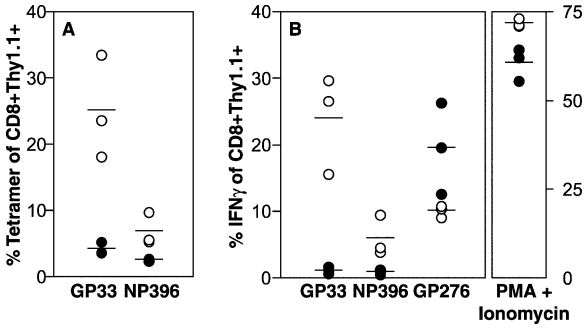

Figure 5. Tolerance Is Not Due to Promiscuous CD11c Promoter Activity Resulting in Fratricide

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 −7 and were infected with 100 pfu LCMV-Armstrong on d 0, and the LCMV-specific CTL response was analyzed on d 12 by tetramer staining (A) and by ICS (B). Each group contained three mice.

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.

We did not find proliferation of endogenous naive CTL precursors as a result of antigen presentation by resting DC. This is in contrast with data published by others (Belz et al., 2002; Bonifaz et al., 2002; Hawiger et al., 2001; Hernandez et al., 2001; Kreuwel et al., 2002; Kurts et al., 1997; Liu et al., 2002; Scheinecker et al., 2002), 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 cannot conclude from our data whether or to what 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., 2001; Kurts et al., 1997). However, other studies demonstrated that immature (Dhodapkar et al., 2001; Dhodapkar and Steinman, 2002; Hugues et al., 2002) or partially mature (Menges et al., 2002; King et al., 2001) DC induce regulatory T cells that suppress T cell function by IL-10 (Dhodapkar et al., 2001; Dhodapkar and Steinman, 2002; 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 frequencies of endogenous, naive 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 naive, endogenous CTL to expand and to develop effector functions, and that the very same DC in a 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 cannot be broken by a strong stimulus, such as infection with a replicating virus, or restimulation in vitro.

Experimental Procedures

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, pCMV33.396.497eGFP, 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 pCMV33.396.497eGFP, located in the multiple cloning site 5′ to the coding sequences. The resulting plasmid, pCMVSTOPL33.396.497eGFP, was digested with NotI, blunted, and 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′TCGAATCTTTCAGCCACAGAATGGGCAGTTCATTCA CTCTAGAATTATTTGCCCCATGTACGCCCGCGTGGATGGGCC3′;

396.497.2, 5′CATCCACGCGGGCGTACATGGGGCAAATAATTCTAG AGTGAATGAACTGCCCATTCTGTGGCTGAAAGAT3′. 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). 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) (Buchmeier et al., 1980). LCMV was propagated on L929 cells at a low multiplicity of infection (moi).

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, OH). 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

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 hr 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 with 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 hr after injection with TAM, and at least until 75 hr after TAM injection. The number of CD11c$^+$GFP$^+$ cells in spleen, lymph nodes, and thymus peaked around 40 hr (data not shown).

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).

Intracellular Staining for Interferon-γ (ICS)

Splenocytes were incubated for 6 hr 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 50 U/ml recombinant mouse IL-2 with irradiated, thioglycollate-elicited peritoneal macrophages, that were loaded with 10^{-8} M of the relevant peptide. Three-fold 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 hr chromium release assay. The percentage of specific lysis was calculated as follows: % specific release = (experimental release – spontaneous release) ÷ (maximal release – spontaneous release) × 100%.

Protection Assay

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

Acknowledgments

We thank Kathrin Tschannen (Inst. Exp. Immunol., Zürich) for the generation of tetramers, Tobias Junt (Inst. Exp. Immunol.) for discussions and reading of the manuscript, and Rolf Zinkernagel and Hans Hengartner (Inst. Exp. Immunol.) for continued support. We are indebted to Jörg Fehr, Werner Kehrli, and Flora Nicholls (Biologisches Zentrallabor, Zürich) for excellent animal husbandry, and to Stefan Freigang, Marianne Martinic, and Kathy McCoy (Inst. Exp. Immunol.) for assistance with experiments. We thank Bernd Arnold and Günter Hämmerling (DKFZ, Heidelberg) for the RAGE% mice, Antonius Rolink (Biozentrum, Basel) for the anti-CD40 producing hybridoma FGK45.5, Pierre Chambon (IGBMC, Strasbourg) for the CreER$_T$ encoding plasmid, Andrew Matus (FMI, Basel) for the β-actin promoter encoding plasmid, and Thomas Brocker (Munich) for the plasmid containing the CD11c promoter. This study was financially supported by the Max Clöetta Foundation Zürich, by the Swiss National Science Foundation, by the European Community (QLG1-CT-1999-00202), and by the Swiss Bundesamt für Bildung und Wissenschaft (BBW).

Received: January 22, 2003
Accepted: March 26, 2003
Published: May 13, 2003

References

Altman, J.D., Moss, P.A., Goulder, P.J., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94–96.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245–252.

Battegay, M., Cooper, S., Althage, A., Banziger, J., Hengartner, H., and Zinkernagel, R.M. (1991). Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. J. Virol. Methods *33*, 191–198.

Belz, G.T., Behrens, G.M., Smith, C.M., Miller, J.F., Jones, C., Lejon,

K., Fathman, C.G., Mueller, S.N., Shortman, K., Carbone, F.R., and Heath, W.R. (2002). The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. J. Exp. Med. *196*, 1099–1104.

Bennett, S.R., Carbone, F.R., Karamalis, F., Flavell, R.A., Miller, J.F., and Heath, W.R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature *393*, 478–480.

Blattman, J.N., Antia, R., Sourdive, D.J., Wang, X., Kaech, S.M., Murali-Krishna, K., Altman, J.D., and Ahmed, R. (2002). Estimating the precursor frequency of naive antigen-specific CD8 T cells. J. Exp. Med. *195*, 657–664.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M.C., and Steinman, R.M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J. Exp. Med. *196*, 1627–1638.

Bourgeois, C., Rocha, B., and Tanchot, C. (2002). A role for CD40 expression on $CD8^+$ T cells in the generation of $CD8^+$ T cell memory. Science *297*, 2060–2063.

Brocker, T., Riedinger, M., and Karjalainen, K. (1997). Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. J. Exp. Med. *185*, 541–550.

Buchmeier, M.J., Welsh, R.M., Dutko, F.J., and Oldstone, M.B. (1980). The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv. Immunol. *30*, 275–331.

Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature *388*, 782–787.

Constien, R., Forde, A., Liliensiek, B., Grone, H.J., Nawroth, P., Hammerling, G., and Arnold, B. (2001). Characterization of a novel EGFP reporter mouse to monitor Cre recombination as demonstrated by a Tie2 Cre mouse line. Genesis *30*, 36–44.

Dhodapkar, M.V., and Steinman, R.M. (2002). Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans. Blood *100*, 174–177.

Dhodapkar, M.V., Steinman, R.M., Sapp, M., Desai, H., Fossella, C., Krasovsky, J., Donahoe, S.M., Dunbar, P.R., Cerundolo, V., Nixon, D.F., and Bhardwaj, N. (1999). Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J. Clin. Invest. *104*, 173–180.

Dhodapkar, M.V., Steinman, R.M., Krasovsky, J., Munz, C., and Bhardwaj, N. (2001). Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J. Exp. Med. *193*, 233–238.

Diehl, L., den Boer, A.T., Schoenberger, S.P., van der Voort, E.I., Schumacher, T.N., Melief, C.J., Offringa, R., and Toes, R.E. (1999). CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. Nat. Med. *5*, 774–779.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. Proc. Natl. Acad. Sci. USA *93*, 10887–10890.

Gallucci, S., Lolkema, M., and Matzinger, P. (1999). Natural adjuvants: endogenous activators of dendritic cells. Nat. Med. *5*, 1249–1255.

Guermonprez, P., Fayolle, C., Rojas, M.J., Rescigno, M., Ladant, D., and Leclerc, C. (2002). In vivo receptor-mediated delivery of a recombinant invasive bacterial toxoid to CD11c+CD8 alpha-CD11b(high) dendritic cells. Eur. J. Immunol. *32*, 3071–3081.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J.V., Steinman, R.M., and Nussenzweig, M.C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J. Exp. Med. *194*, 769–779.

Hernandez, J., Aung, S., Redmond, W.L., and Sherman, L.A. (2001). Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. J. Exp. Med. *194*, 707–717.

Hugues, S., Mougneau, E., Ferlin, W., Jeske, D., Hofman, P., Homann, D., Beaudoin, L., Schrike, C., Von Herrath, M., Lehuen, A., and Glaichenhaus, N. (2002). Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. Immunity *16*, 169–181.

Huleatt, J.W., and Lefrancois, L. (1995). Antigen-driven induction of CD11c on intestinal intraepithelial lymphocytes and CD8+ T cells in vivo. J. Immunol. *154*, 5684–5693.

Inaba, K., Inaba, M., Naito, M., and Steinman, R.M. (1993). Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. J. Exp. Med. *178*, 479–488.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., et al. (2002). In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity *17*, 211–220.

Kaisho, T., and Akira, S. (2001). Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. Trends Immunol. *22*, 78–83.

King, C., Mueller, H.R., Malo, C., Murali-Krishna, K., Ahmed, R., King, E., and Sarvetnick, N. (2001). IL-4 acts at the locus of the antigen-presenting dendritic cell to counter-regulate cytotoxic CD8+ T cell responses. Nat. Med. *7*, 206–214.

Kreuwel, H.T., Aung, S., Silao, C., and Sherman, L.A. (2002). Memory CD8(+) T cells undergo peripheral tolerance. Immunity *17*, 73–81.

Kurts, C., Kosaka, H., Carbone, F.R., Miller, J.F., and Heath, W.R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. J. Exp. Med. *186*, 239–245.

Legge, K.L., Gregg, R.K., Maldonado-Lopez, R., Li, L., Caprio, J.C., Moser, M., and Zaghouani, H. (2002). On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. J. Exp. Med. *196*, 217–227.

Liu, K., Iyoda, T., Saternus, M., Kimura, Y., Inaba, K., and Steinman, R.M. (2002). Immune tolerance after delivery of dying cells to dendritic cells in situ. J. Exp. Med. *196*, 1091–1097.

Ludin, B., Doll, T., Meili, R., Kaech, S., and Matus, A. (1996). Application of novel vectors for GFP-tagging of proteins to study microtubule-associated proteins. Gene *173*, 107–111.

Menges, M., Rossner, S., Voigtlander, C., Schindler, H., Kukutsch, N., Bogdan, C., Erb, K., Schuler, G., and Lutz, M. (2002). Repetitive injections of dendritic cells matured with tumor necrosis factor-alpha induce antigen-specific protection of mice from autoimmunity. J. Exp. Med. *195*, 15–21.

Morgan, D.J., Liblau, R., Scott, B., Fleck, S., McDevitt, H.O., Sarvetnick, N., Lo, D., and Sherman, L.A. (1996). CD8(+) T cell-mediated spontaneous diabetes in neonatal mice. J. Immunol. *157*, 978–983.

Morgan, D.J., Kreuwel, H.T., and Sherman, L.A. (1999). Antigen concentration and precursor frequency determine the rate of CD8+ T cell tolerance to peripherally expressed antigens. J. Immunol. *163*, 723–727.

Oukka, M., Cohen-Tannoudji, M., Tanaka, Y., Babinet, C., and Kosmatopoulos, K. (1996). Medullary thymic epithelial cells induce tolerance to intracellular proteins. J. Immunol. *156*, 968–975.

Pierre, P., Turley, S.J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R.M., and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. Nature *388*, 787–792.

Probst, H.C., Dumrese, T., and van den Broek, M.F. (2002). Cutting edge: competition for APC by CTLs of different specificities is not functionally important during induction of antiviral responses. J. Immunol. *168*, 5387–5391.

Roake, J.A., Rao, A.S., Morris, P.J., Larsen, C.P., Hankins, D.F., and Austyn, J.M. (1995). Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. J. Exp. Med. *181*, 2237–2247.

Rolink, A., Melchers, F., and Andersson, J. (1996). The SCID but not the RAG-2 gene product is required for S mu-S ϵ heavy chain class switching. Immunity *5*, 319–330.

Ruedl, C., Kopf, M., and Bachmann, M.F. (1999). CD8(+) T cells mediate CD40-independent maturation of dendritic cells in vivo. J. Exp. Med. *189*, 1875–1884.

Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C.R., Qin, S., and Lanzavecchia, A. (1998). Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. Eur. J. Immunol. *28*, 2760–2769.

Sauer, B. (1993). Manipulation of transgenes by site-specific recombination: use of Cre recombinase. Methods Enzymol. *225*, 890–900.

Scheinecker, C., McHugh, R., Shevach, E.M., and Germain, R.N. (2002). Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. J. Exp. Med. *196*, 1079–1090.

Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R., and Melief, C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. Nature *393*, 480–483.

Schuurhuis, D.H., Laban, S., Toes, R.E., Ricciardi-Castagnoli, P., Kleijmeer, M.J., van der Voort, E.I., Rea, D., Offringa, R., Geuze, H.J., Melief, C.J., and Ossendorp, F. (2000). Immature dendritic cells acquire CD8(+) cytotoxic T lymphocyte priming capacity upon activation by T helper cell-independent or -dependent stimuli. J. Exp. Med. *192*, 145–150.

Sotomayor, E.M., Borrello, I., Tubb, E., Rattis, F.M., Bien, H., Lu, Z., Fein, S., Schoenberger, S., and Levitsky, H.I. (1999). Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through in vivo ligation of CD40. Nat. Med. *5*, 780–787.

van der Most, R.G., Sette, A., Oseroff, C., Alexander, J., Murali-Krishna, K., Lau, L.L., Southwood, S., Sidney, J., Chesnut, R.W., Matloubian, M., and Ahmed, R. (1996). Analysis of cytotoxic T cell responses to dominant and subdominant epitopes during acute and chronic lymphocytic choriomeningitis virus infection. J. Immunol. *157*, 5543–5554.

Wang, B., Norbury, C.C., Greenwood, R., Bennink, J.R., Yewdell, J.W., and Frelinger, J.A. (2001). Multiple paths for activation of naive CD8+ T cells: CD4-independent help. J. Immunol. *167*, 1283–1289.