

Resting dendritic cells induce peripheral CD8⁺ T cell tolerance through PD-1 and CTLA-4

Hans Christian Probst¹, Kathy McCoy¹, Taku Okazaki², Tasuku Honjo² & Maries van den Broek¹

T cells recognizing self proteins exist without causing autoimmunity in healthy individuals. These autoreactive T cells are kept in check by peripheral tolerance. Using a model for peripheral CD8⁺ T cell tolerance resulting from antigen presentation by resting dendritic cells *in vivo*, we show here that CD8⁺ T cell tolerance operates through T cell-intrinsic mechanisms such as deletion or functional inactivation. Peripheral CD8⁺ T cell tolerance depended on signaling via the costimulatory molecule PD-1, as an absence of PD-1 converted tolerance induction into priming. Blocking of the costimulatory molecule CTLA-4 resulted in impaired tolerance and enhanced the effect of the absence of PD-1, suggesting that PD-1 and CTLA-4 act synergistically. Thus PD-1 and CTLA-4 are crucial molecules for peripheral CD8⁺ T cell tolerance induced by resting dendritic cells.

Self-tolerance of T cells is induced and maintained in different compartments of the immune system¹. Developing T cells can be clonally deleted in the thymus as a result of negative selection, which requires the presence of relevant autoantigens. Many tissue-specific autoantigens are present in the thymus², probably because of random derepression of genes encoding tissue-specific antigens³, regulated by the transcription factor Aire^{4,5}. However, it is unlikely that every autoantigen will be present in sufficient amounts to guarantee the deletion of all self-reactive T cells, and it has been shown that healthy individuals have autoreactive T cells in the periphery⁶. Peripheral tolerance is therefore a mechanism supplementary to central tolerance and is presumably crucial to prevent autoimmunity⁷. The mechanisms responsible for peripheral CD8⁺ T cell tolerance can be divided into those acting directly on the responding T cells, such as inactivation or deletion of specific T cells^{7–9} (T cell intrinsic), and those that act through additional cells or factors, such as regulatory T cells or suppressive cytokines¹⁰ (T cell extrinsic).

Coinhibitory molecules of the CD28 family are essential for the maintenance of T cell homeostasis and self-tolerance. Mice deficient in cytotoxic T lymphocyte (CTL)-associated antigen 4 (CTLA-4; CD152) develop lethal autoimmunity and lymphoproliferation^{11,12}, suggesting that the maintenance of T cell tolerance *in vivo* depends on ligation of CTLA-4, a molecule homologous to CD28, by CD80 (B7-1) and CD86 (B7-2)^{13,14}. CTLA-4 is rapidly expressed by T cells after T cell receptor ligation¹⁵, but there is evidence that naive T cells also express CTLA-4 to some extent¹⁶. Another molecule that is presumably involved in the control of T cell responsiveness is programmed cell death 1 (PD-1)¹⁷, a member of the immunoglobulin superfamily that is expressed by T cells, B cells and myeloid cells¹⁸. Evidence for its possible involvement in the negative regulation of immune responses came from PD-1-deficient mice, which spontaneously develop

autoimmune disorders such as glomerulonephritis¹⁹ and lethal dilated cardiomyopathy²⁰. The ligands for PD-1 are B7-H1 (PD-L1)²¹ and B7-DC (PD-L2)²², which are expressed by DCs, macrophages, lymphocytes and nonlymphoid tissues^{23,24}.

Dendritic cells (DCs) are crucial in the induction of T cell immunity as well as in peripheral T cell tolerance. Interaction of naive T cells with resting, antigen-presenting DCs results in antigen-specific T cell tolerance, whereas activated DCs induce priming^{9,25–29}. A system that allows inducible antigen presentation by resting or by activated DCs *in vivo* has been described: injection of double-transgenic DIETER mice ('DC-specific inducible expression of T cell epitopes by recombination' mice) with tamoxifen induces the presentation of three transgenic CTL epitopes by 5% of all DCs, which results in robust peripheral T cell tolerance if the DCs are resting and in protective immunity if the DCs are activated *in vivo* by coinjection of agonistic CD40 antibodies²⁵. Here we use DIETER mice to investigate molecular mechanisms involved in the induction of peripheral CD8⁺ T cell tolerance *in vivo*. We report that peripheral CD8⁺ T cell tolerance induced by resting DCs is T cell intrinsic and depends on the engagement of PD-1 and CTLA-4.

RESULTS

Resting DCs induce peripheral CD8⁺ T cell tolerance

Injection of DIETER mice with 2 mg tamoxifen results in the presentation of three transgenic CTL epitopes by DCs: lymphocytic choriomeningitis virus (LCMV) glycoprotein, amino acids 33–41 (GP(33–41)), LCMV nucleoprotein, amino acids 396–404 (NP(396–404)), and β -galactosidase, amino acids 497–504. Inducible antigen presentation by DCs in DIETER mice is achieved by the expression of a tamoxifen-inducible Cre recombinase under the control of the mouse CD11c promoter. Because this promoter is also active on activated

¹Institute of Experimental Immunology, University Hospital Zurich, Switzerland. ²Department of Medical Chemistry, Kyoto University, Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto, Japan. Correspondence should be addressed to M.B. (maries@pathol.unizh.ch).

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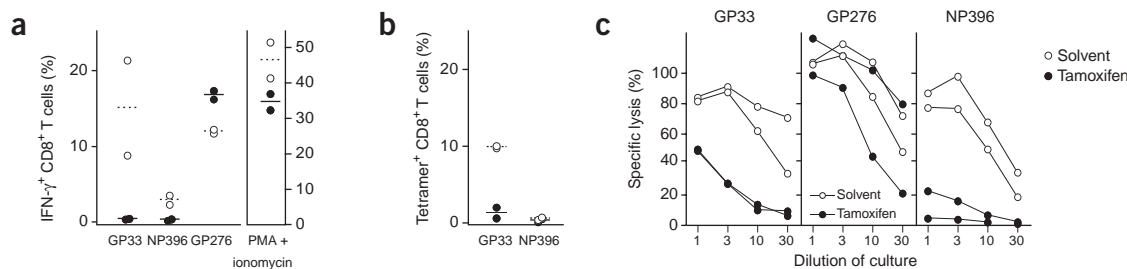


Figure 1 Antigen presentation by resting DCs induces antigen-specific CD8⁺ T cell tolerance. RAG-DIETER-B6 mixed bone marrow chimeras were injected intraperitoneally at day -7 with tamoxifen (solid lines, means) or with solvent (dashed lines, means). They were infected intravenously on day 0 with LCMV-WE, and the LCMV-induced CTL response was measured on day 12 in the spleen by intracellular staining for IFN- γ after restimulation *in vitro* with LCMV-derived peptides (left) for quantification of peptide-specific CD8⁺ T cell responses, or with phorbol 12-myristate 13-acetate (PMA) plus ionomycin (right) for quantification of the total LCMV-specific CD8⁺ T cell response (a), by tetramer staining (b) and by a cytotoxicity assay after restimulation *in vitro*²⁵ (c). Lysis of EL-4 targets without peptide was less than 10% of the specific lysis. Spontaneous lysis of targets was less than 15%. Data are one representative experiment of four. Symbols or curves represent individual mice. GP33, GP(33-41); GP276, GP(276-286); NP396, NP(396-404).

T cells³⁰, we wanted to exclude the possibility of an influence of antigen presentation by lymphocytes in this system. Therefore, we bred DIETER mice onto a recombination activating gene-deficient background (RAG-DIETER). We then generated mixed bone marrow chimeras by injecting a 2:1 mixture of RAG-DIETER and C57BL/6 (B6) wild-type bone marrow into lethally irradiated B6 mice. In these chimeras, all lymphocytes were derived from B6 bone marrow, whereas DCs were derived from both B6 and RAG-DIETER bone marrow. At 6 weeks after reconstitution, we injected RAG-DIETER-B6 mixed bone marrow chimeras with 2 mg tamoxifen or with solvent (day -7) and infected them 7 d later (day 0) with 100 plaque-forming units (PFU) of LCMV, strain WE (LCMV-WE). LCMV infection of B6 (H-2^b) mice induces CTLs specific for three immunodominant epitopes: GP(33-41), GP(276-286) and NP(396-404)³¹, two of which, GP(33-41) and NP(396-404), are presented by DIETER DCs after tamoxifen injection. We analyzed priming by LCMV 12 d after infection by intracellular staining for interferon- γ (IFN- γ ; Fig. 1a), by tetramer staining (Fig. 1b) and by a CTL assay after restimulation *in vitro*, which is a highly sensitive 'readout' that allows the detection of very low numbers of antigen-specific CD8⁺ T cells (Fig. 1c). We

found greatly reduced GP(33-41)-specific and NP(396-404)-specific CTLs in tamoxifen-treated, LCMV-infected RAG-DIETER-B6 mixed bone marrow chimeras compared with solvent-treated mice, but a normal GP(276-286) response. This result shows that antigen presentation by resting DCs resulted in tolerance of specific CD8⁺ T cells, which could not be broken by subsequent infection with LCMV, confirming published results in a situation in which antigen presentation on lymphocytes was formally excluded²⁵.

Resting DCs induce tolerance by a T cell-intrinsic mechanism

Two possible mechanisms for the induction of peripheral tolerance of CD8⁺ T cells after antigen presentation by resting DCs can be envisaged. Tolerance induction could be a T cell-extrinsic process that involves regulatory cells, or it could be a T cell-intrinsic process such as the induction of unresponsiveness or deletion of the T cells specific for the CD8⁺ T cell epitopes presented by resting DCs. The strict antigen specificity of CD8⁺ T cell tolerance in DIETER mice provides strong evidence for a T cell-intrinsic mechanism of tolerance induction DCs (Fig. 1). Nevertheless, to address this issue formally, we analyzed whether CD8⁺ T cell tolerance induced in DIETER mice

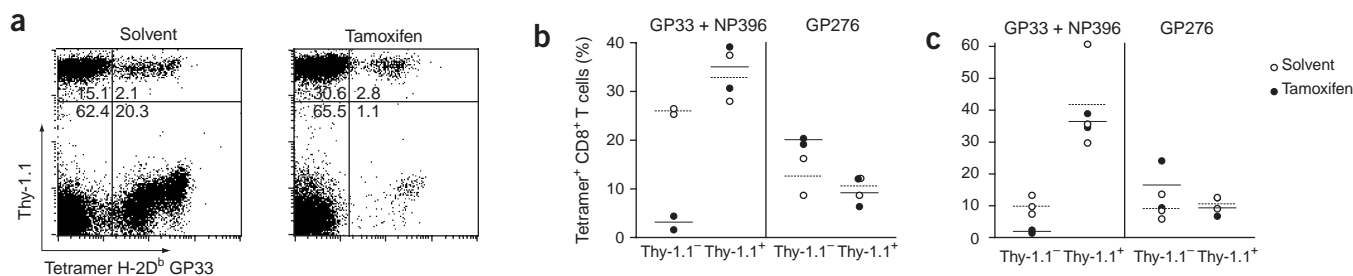
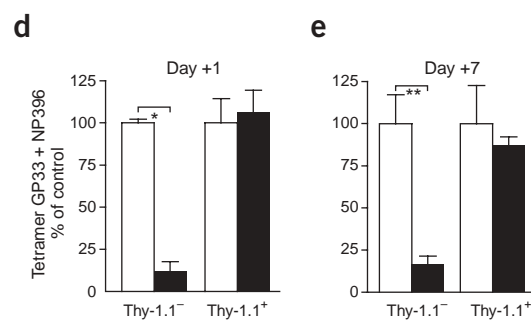


Figure 2 CD8⁺ T cell tolerance induced by antigen-presenting resting DCs operates through T cell-intrinsic mechanisms. (a-c) RAG-DIETER-B6 mixed bone marrow chimeras were injected intraperitoneally at day -12 with tamoxifen (b,c: solid lines, means) or with solvent (b,c: dashed lines, means). At day 0, naive, allotypically different splenocytes were adoptively transferred into tolerized or control mixed bone marrow chimeras. Mice were infected with 1×10^2 PFU LCMV-WE at day +1 (a,b) or day +7 (c), and the expansion of endogenous (Thy-1.1⁻) and of transferred (Thy-1.1⁺) CD8⁺ T cell populations specific for LCMV epitopes encoded by transgenes (GP(33-41) and NP(396-404)) as well as for the control epitope (GP(276-286)) was measured by tetramer staining 12 d after infection. Data are one representative experiment of two. Symbols represent individual mice. Numbers in dot plots in a indicate the percentage of cells in each quadrant. (d,e) Data from b and c, respectively, expressed relative to the mean of the respective solvent-treated groups, which were set to 100%. Error bars represent the s.d. *, $P < 0.005$; **, $P < 0.05$.



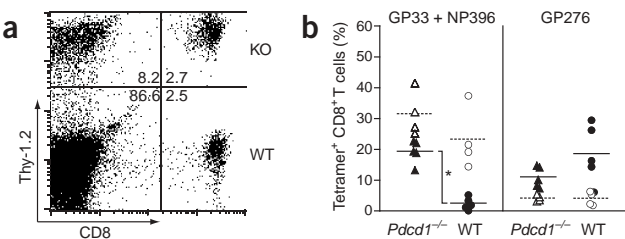


Figure 3 An absence of PD-1 engagement impairs peripheral tolerance by resting DCs. **(a)** Reconstitution of 'RAG-DIETER (Thy-1.2) + B6-Thy-1.1 + PD-1-deficient (Thy-1.2) → B6-Thy-1.1' mixed bone marrow chimeras was assessed 6 weeks after transplantation by staining of blood lymphocytes for CD8 and Thy-1.2. Numbers indicate the percentage of cells in each quadrant. **(b)** Chimeras were injected intraperitoneally at day -7 with tamoxifen (filled symbols; solid lines, means) or with solvent (open symbols; dashed lines, means). They were infected intravenously on day 0 with LCMV-WE, and the LCMV-induced response of wild-type CD8⁺ (Thy-1.1) and of PD-1-deficient CD8⁺ (Thy-1.2) cells specific for transgene-encoded epitopes (GP(33–41) and NP(396–404)) as well as for the control epitope (GP(276–286)) was measured in the spleen on day 12 by tetramer staining. KO and *Pdc1*^{-/-}, PD-1-deficient; WT, wild-type. Data are one representative experiment of three. Symbols represent individual mice. *, *P* < 0.0001.

affected the responsiveness of naive CD8⁺ T cells transferred into tolerant DIETER mice.

We injected naive Thy-1.1⁺ T cells into Thy-1.2⁺ RAG-DIETER-B6 chimeras that were tolerized by the injection of tamoxifen or solvent 12 d before and then analyzed whether the transferred T cells became unresponsive as a result of being in a tolerant environment. By waiting 12 d after tamoxifen injection before transferring naive splenocytes, we avoided tolerization of transferred T cells by resting, antigen-presenting DIETER DCs, because the half-life of DCs is limited^{32,33}, as is the serum half-life of tamoxifen³⁴. We infected mice with 100 PFU LCMV-WE 1 d or 7 d after transfer of naive splenocytes and analyzed priming by LCMV using tetramer staining. As expected, endogenous (Thy-1.2⁺) CD8⁺ T cell populations specific for the transgene-encoded epitopes did not expand in tamoxifen-treated RAG-DIETER-B6 mice because of tolerance, whereas those specific for the control LCMV epitope GP(276–286) did (Fig. 2). In contrast, LCMV infection induced normal expansion of adoptively transferred Thy-1.1⁺ CD8⁺ T cell populations specific for all three LCMV-derived epitopes in tamoxifen- and in solvent-treated RAG-DIETER-B6 mice, independently of whether they had been exposed to a tolerant environment for 1 d (Fig. 2a,b,d) or for 7 d (Fig. 2c,e). These data show that peripheral tolerance lasts for at least 19 d. Analysis of tolerance beyond this time point in the presence of an intact thymus is complicated by the appearance of naive T cells from the thymus. Thus, CD8⁺ T cell tolerance induced by resting DCs operates through a T cell–intrinsic mechanism whereby the total T cell repertoire is functionally depleted of T cells specific for the epitopes presented by the resting DCs.

Peripheral CD8⁺ T cell tolerance DCs depends on PD-1

We next sought to determine the molecular mechanism by which resting antigen-presenting DCs induce unresponsiveness or deletion of antigen-specific CD8⁺ T cells. Based on the autoimmune phenotype of knockout mice^{11,12,19,20} and on data obtained with blocking reagents^{13,14,21,22,35}, we considered the involvement of PD-1 and CTLA-4 in transmission of the negative signals from resting DCs.

To investigate the function of PD-1 in peripheral tolerance induction by resting DCs, we made mixed bone marrow chimeras of RAG-DIETER and PD-1-deficient mice. PD-1-deficient mice develop

a spontaneous lupus-like autoimmune disease¹⁹. Although disease in PD-1-deficient mice does not develop before 6 months of age, younger PD-1-deficient mice may nonetheless have some subclinical inflammation and T cell activation, which could lead to DC activation and to inhibition of tolerance induction in PD-1-deficient–RAG-DIETER mixed bone marrow chimeras. To exclude the possibility of such nonspecific effects of the PD-1 deficiency, we included bone marrow cells from wild-type B6 (Thy-1.1⁺) mice in the mixed bone marrow chimeras. This mixture allowed us to analyze the response of PD-1-deficient (Thy-1.2⁺) and wild-type (Thy-1.1⁺) CD8⁺ T cells in the same mouse. We lethally irradiated B6–Thy-1.1⁺ recipient mice and reconstituted them with 70% RAG-DIETER, 15% B6–Thy-1.1 and 15% PD-1-deficient (Thy-1.2⁺) bone marrow. The resulting chimeras expressed the DIETER transgene on 70% of their DCs, whereas the T cells were derived in equal proportions from PD-1-deficient (Thy-1.2) and B6–Thy-1.1 bone marrow, as shown by flow cytometry of peripheral blood lymphocytes 6 weeks after bone marrow transplantation (Fig. 3a). At 6 weeks after reconstitution, we injected chimeras at day -7 with tamoxifen to induce antigen presentation by resting DCs or as control with solvent. At d 0, we infected mice with 100 PFU LCMV-WE, and 12 d later we analyzed the LCMV-specific CTL response against the DIETER transgene–encoded epitopes GP(33–41) and NP(396–404) and against the additional LCMV-derived epitope GP(276–286) using major histocompatibility complex (MHC) class I tetramers.

Analysis of the response in the PD-1-deficient compartment (Thy-1.1⁻) showed that tolerance was impaired in CD8⁺ T cells lacking the PD-1 receptor, indicating that PD-1 is important in the induction of peripheral CD8⁺ T cell tolerance by resting DCs (Fig. 3b, left). We found normal tolerance induction in the wild-type compartment (Thy-1.1⁺) of the same individual mice, excluding the possibility that impaired tolerance in CD8⁺ T cells lacking PD-1 was due to a nonspecific effect resulting from PD-1 deficiency. As described before²⁵, an enhanced response against GP(276–286) compensated for the reduced response against GP(33–41) and NP(396–404) in the case of both wild-type and PD-1-deficient CD8⁺ T cells in tamoxifen-treated mice (Fig. 3b, right).

CTLA-4 contributes to peripheral CD8⁺ T cell tolerance induction

To investigate the involvement of CTLA-4 in peripheral CD8⁺ T cell tolerance induced by resting DCs, we used a monoclonal antibody that

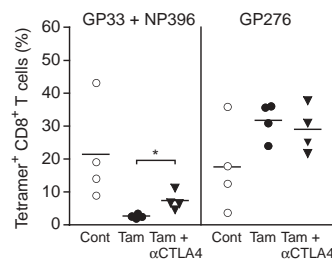


Figure 4 Blocking of CTLA-4 diminishes peripheral CD8⁺ T cell tolerance induced by resting DCs. 'RAG-DIETER + B6' mixed bone marrow chimeras were injected intraperitoneally at day -7 with tamoxifen (Tam; filled circles), with tamoxifen plus anti-CTLA-4 (Tam + αCTLA4; filled triangles) or with solvent (Cont; open circles). They were infected intravenously on day 0 with LCMV-WE, and the LCMV-induced CTL response specific for epitopes encoded by transgenes (GP(33–41) and NP(396–404)) as well as for the control epitope (GP(276–286)) was measured in the spleen on day 12 by tetramer staining. Data are one representative experiment of four. Symbols represent individual mice. *, *P* < 0.05.

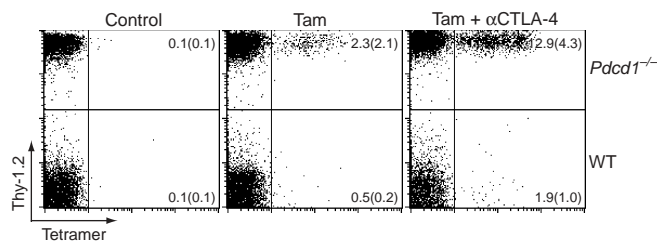


Figure 5 An absence of signaling through PD-1 and CTLA-4 converts tolerance induction into priming. 'RAG-DIETER (Thy-1.2) + B6-Thy-1.1 + PD-1-deficient (Thy-1.2) → B6-Thy-1.1' mixed bone marrow chimeras were injected intraperitoneally at day -7 with tamoxifen or with tamoxifen plus anti-CTLA-4 or were left untreated. At d 0, the transgene-specific response of endogenous wild-type CD8⁺ (Thy-1.1⁺) and of endogenous PD-1-deficient CD8⁺ (Thy-1.2⁺) cells was measured in the blood. Data from one of three mice per group are shown; numbers in quadrants represent the mean (\pm s.d.) of three mice. Data are one representative experiment of three.

blocks signaling through CTLA-4 *in vivo*. At day -7, we injected RAG-DIETER-B6 mixed bone marrow chimeras with tamoxifen, with tamoxifen plus 0.5 mg hamster antibody to CTLA-4 (anti-CTLA-4; 4F10) or with solvent and infected the mice at day 0 with 100 PFU LCMV-WE. Here we used mice treated with tamoxifen rather than with tamoxifen plus hamster immunoglobulin G (IgG) as a control; however, we excluded the possibility that injection of 4F10 resulted in activation of DCs (Methods).

Blocking of CTLA-4 at the time of tolerance induction resulted in a moderate but consistent reduction in tolerance (Fig. 4, left). The responses against the control epitope GP(276–286) were comparable in all three situations (Fig. 4, right). Thus, CTLA-4 is also essential for complete tolerance induction after the presentation of CTL epitopes on resting DCs.

Lack of PD-1 and CTLA-4 results in priming rather than tolerance

Antigen presentation by resting DCs induces CD8⁺ T cell tolerance only if the responding CD8⁺ T cells can receive signals through PD-1 and CTLA-4. If either of these inhibitory signals were missing, the T cells remained responsive to stimulation by LCMV infection. To determine whether presentation of CTL epitopes by resting DCs in the absence of these inhibitory signals had left the CD8⁺ T cells naive or

whether it had induced priming, we examined the expansion of specific CTL populations in these circumstances. We prevented either of these inhibitory signals alone or simultaneously. We used mixed chimeras that had been reconstituted with 70% RAG-DIETER, 15% PD-1-deficient and 15% B6-Thy-1.1 bone marrow and injected them with tamoxifen to induce antigen presentation by resting DCs. In some of the mice, we blocked CTLA-4 signaling by injection with 0.5 mg anti-CTLA4 (4F10) together with the tamoxifen treatment. At 7 d after tamoxifen treatment, we analyzed peripheral blood lymphocytes with tetramers for the expansion of antigen-specific CD8⁺ T cell populations. Substantial expansion of antigen-specific CD8⁺ PD-1-deficient T cell populations occurred after tamoxifen treatment, whereas wild-type CD8⁺ T cell populations did not expand (Fig. 5, middle). The expansion of specific CD8⁺ T cell populations in the PD-1-deficient compartment was significantly enhanced ($P < 0.05$) when CTLA-4 signaling was also blocked, suggesting an additive effect of signals through PD-1 and CTLA-4 in CD8⁺ T cell tolerance (Fig. 5, right). We found only limited expansion of specific CTL populations in wild-type CD8⁺ T cells of mice treated with tamoxifen plus anti-CTLA-4. Thus, antigen presentation by resting DCs induced priming of endogenous CD8⁺ T cells in the absence of PD-1 signaling and additional blocking of CTLA-4 substantially enhanced this priming. Blocking CTLA-4 in the presence of normal PD-1 signaling resulted in weak but consistent priming.

To examine whether antigen presentation by resting DCs in the absence of PD-1 and CTLA-4 signaling resulted only in expansion of specific CD8⁺ T cell populations or in genuine CTL priming, we repeated the experiment described above and treated bone marrow chimeras (70% RAG-DIETER, 15% PD-1-deficient and 15% B6-Thy-1.1 bone marrow) with tamoxifen plus anti-CTLA-4 or with tamoxifen plus control hamster IgG. We monitored the expansion of CD8⁺ T cell populations specific for all three transgene-encoded epitopes (Fig. 6a) and analyzed their effector function using intracellular staining for IFN- γ after 6 h of restimulation with the antigenic peptide *in vitro* (Fig. 6b). We found that the expanded transgene-specific CD8⁺ T cell populations indeed produced IFN- γ in an antigen-specific manner. As an additional test of the effector function of the CTL populations that had expanded in the absence of inhibitory costimulation, at 7 d after tamoxifen and antibody treatment we infected these mixed bone marrow chimeras with 100 PFU LCMV-WE. We measured virus titers 4 d after infection and found that mixed bone

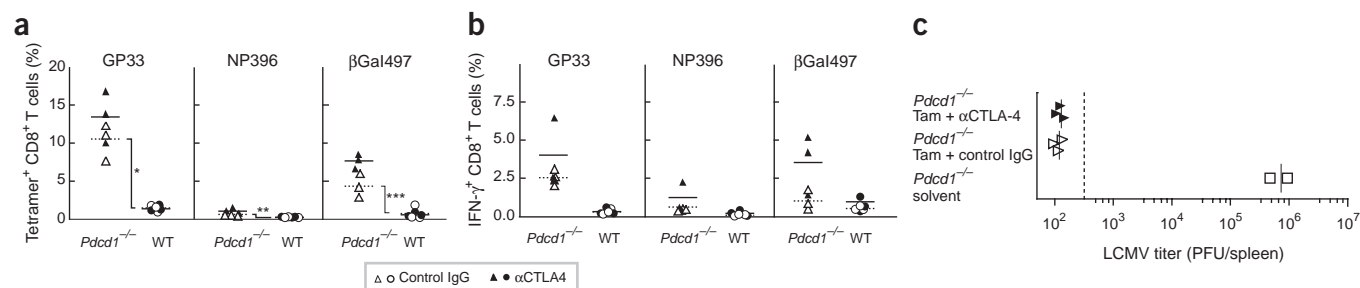


Figure 6 Expansion of transgene-specific CD8⁺ T cell populations in the absence of signaling through PD-1 and CTLA-4 represents genuine priming of effector CTLs. 'RAG-DIETER (Thy-1.2) + B6-Thy-1.1 + PD-1-deficient (Thy-1.2) → B6-Thy-1.1' mixed bone marrow chimeras were injected intraperitoneally at day -7 with tamoxifen plus anti-CTLA-4 (α CTLA4; solid lines, mean) or with tamoxifen plus control hamster IgG (Control IgG; dashed lines, mean). At day 0, the transgene-specific response of endogenous wild-type CD8⁺ (Thy-1.1⁺) and of endogenous PD-1-deficient CD8⁺ (Thy-1.2⁺) cells was measured in the spleen. The transgene-specific CD8⁺ response was determined by tetramer staining (a) and by intracellular staining for IFN- γ after restimulation *in vitro* with the respective peptide (b). β Gal497, β -galactosidase, amino acids 497–504. (c) Functional activity of the expanded LCMV-specific (GP(33–41) and NP(396–404)) CD8⁺ T cell populations, tested *in vivo* by an antiviral protection assay. Mice were challenged with LCMV-WE 7 d after administration of tamoxifen and viral titers were determined in the spleen 4 d later as described⁵⁶. Dashed vertical line indicates the detection limit of the assay. Data are one representative experiment of two. Symbols represent individual mice. *, $P < 0.005$; **, $P < 0.01$; ***, $P < 0.05$.

marrow chimeras treated with tamoxifen or with tamoxifen plus anti-CTLA-4 had controlled LCMV to undetectable levels, whereas untreated mixed bone marrow chimeras contained large amounts of virus (Fig. 6c). Thus, antigen presentation by resting DCs induces protective immunity if signaling through PD-1 alone or PD-1 and CTLA-4 is absent.

DISCUSSION

The mechanisms that underlie the induction of peripheral T cell tolerance by antigen-presenting resting DCs remain elusive. Both T cell-extrinsic mechanisms, such as the induction of negatively regulating T cells^{36–38} or cytokines³⁹, as well as T cell-intrinsic mechanisms, such as deletion or functional inactivation of specific T cells^{40,41}, have been suggested. A model has been established, based on inducible presentation of MHC class I-restricted CTL epitopes by resting DCs, to investigate the molecular mechanisms of peripheral tolerance of endogenous CD8⁺ T cells²⁵. This model is unique in that it allows the induction of antigen presentation *in vivo* by a fraction of DCs in steady-state conditions. There are various possibilities to explain why DCs are tolerogenic in steady-state conditions: DCs may be tolerogenic by default until they become activated, or they may be kept tolerogenic by active mechanisms such as regulatory T cells⁴² or the phagocytosis of apoptotic cells⁴³. In this model, antigen presentation is induced by approximately 5% of all steady-state CD11c⁺ cells without discrimination or selection, and it is reasonable to assume that all mechanisms that are operative to keep DCs tolerogenic in the steady state in physiological conditions are also at work in this model. Another advantage of this model is the use of endogenous CD8⁺ T cells, which are present as a polyclonal population at physiological low precursor frequencies, as a 'readout'. However, this model might be different from normal physiology in some aspects. For example, although antigen presentation is induced on a limited and presumably physiological number of DCs, these DCs are equally distributed within the body, whereas normally the presentation of tissue-specific self antigens by resting DCs most likely happens in the tissue-draining lymph nodes. In this model, the antigens are inducibly expressed by the DCs itself and therefore directly presented by the classical pathway. This allows restriction of antigen presentation to DCs and precise control of the onset of presentation. In the physiological situation, however, T cells specific for antigens expressed by DCs themselves can be deleted by negative selection in the thymus, whereas peripheral tolerance may be crucial for antigens that are exclusively expressed in peripheral tissues and are cross-presented in the draining lymph nodes^{29,41,44–46}. Despite these characteristics of the model system, the molecular mechanisms of tolerance induction, which are operative in this model, are likely to be identical to those in peripheral CD8⁺ T cell cross-tolerance.

Presentation of GP(33–41) and NP(396–404) by resting DCs resulted in a severely compromised response against GP(33–41) and NP(396–404) and a compensatory high GP(276–286) response after infection with LCMV²⁵. This unambiguously demonstrates that peripheral tolerance of CD8⁺ T cells induced through antigen presentation by resting DCs is strictly antigen specific and strongly challenges the idea of a dominant suppressive mechanism. Formal proof of the recessive, T cell-intrinsic character of peripheral CD8⁺ T cell tolerance came from experiments in which we adoptively transferred naive CD8⁺ T cells into tolerant DIETER mice at a time when the tolerizing stimulus had disappeared, followed by infection with LCMV. Our results are well in line with other experiments showing functional inactivation and peripheral deletion of CD8⁺ T cells^{29,44,46,47} and of CD4⁺ T cells^{28,41,48} as result of the confrontation of naive adoptively

transferred T cells with resting DCs that present the specific peptide antigen. Although there are reports suggesting that negatively regulating T cells mediate peripheral tolerance of CD4⁺ T cells after antigen presentation by resting DCs^{10,37,38}, convincing evidence that such cells are active in peripheral tolerance of CD8⁺ T cells is lacking^{29,41,44–46}.

The autoimmune phenotype of CTLA-4-deficient mice^{11,12} and PD-1-deficient mice^{19,20} and data obtained with blocking reagents^{13,14,21,22,35,49} prompted us to investigate the possible involvement of PD-1 and CTLA-4 in peripheral CD8⁺ T cell tolerance induced by resting DCs. Using mixed bone marrow chimeras, we found that tolerance of PD-1-deficient CD8⁺ T cells was impaired, whereas there was normal tolerance induction in the wild-type CD8⁺ compartment in the same individual mice.

In experiments using T cell receptor-transgenic PD-1-deficient mice, PD-1 was found to have a moderate effect on thymic selection^{50,51}. However, negative selection was found to be more stringent rather than impaired in PD-1-deficient mice⁵¹. In addition, by measuring tetramer-positive cells in blood and spleen and by intracellular staining for IFN- γ , we found no statistically significant difference between the frequencies of transgene-specific (GP(33–41)- and NP(396–404)-specific) wild-type and PD-1-deficient CD8⁺ in solvent-treated 'RAG-DIETER + PD-1-deficient + B6-Thy-1.1' bone marrow chimeras after LCMV infection. Both facts challenge the idea of a contribution of putative altered thymic selection to the loss of peripheral tolerance in PD-1-deficient CD8⁺ T cells. Our data show that in addition to PD-1, the most prominent member of this family of coinhibitory molecules, CTLA-4, is also involved in peripheral tolerance induction by resting DCs. Blocking of CTLA-4 signaling resulted in impaired tolerance induction and enhanced the effect of PD-1 deficiency.

We propose that the balance between negative and positive costimulation, which changes when DCs undergo activation, controls the opposing functions of DCs: T cell priming and tolerance induction. Naive CD8⁺ T cells simultaneously receive, in addition to signals through the T cell receptor, activating and inhibitory costimulation from DCs. If the DC is resting, negative signals through PD-1 and CTLA-4 dominate and tolerance ensues. If the DC is activated, positive signals prevail, resulting in CTL priming. If negative signals through PD-1 and CTLA-4 are absent, resting DCs also induce priming. Priming rather than tolerance might result because the weak activating signals provided by a resting DC are not counterbalanced by inhibitory signals through PD-1 and CTLA-4. Alternatively, few activated DCs, which are present in the steady state even in healthy individuals, could transmit activating signals. Normally these activating signals will be overridden by inhibitory signals through PD-1 and CTLA-4, provided by most DCs, which are resting. If the T cells cannot receive these inhibitory signals through PD-1 and CTLA-4, priming will result. We have shown here that PD-1 and CTLA-4 are central molecules for peripheral CD8⁺ T cell tolerance induction by DC in the steady state. An absence of these molecules leads to T cell priming even in the steady state and may result in autoimmunity.

METHODS

Mice. DIETER mice are double-transgenic offspring of ST33.396 \times CD11cCreERT intercrossing. A single intraperitoneal injection of 2 mg tamoxifen induces expression of three transgenic CTL epitopes on 5–10% of CD11c⁺ cells: GP(33–41) (H-2D^b), NP(396–404) (H-2D^b), and β -galactosidase, amino acids 497–504 (H-2K^b)²⁵. Injection of tamoxifen before LCMV infection did not quantitatively or qualitatively affect the induction of LCMV-specific CD8⁺ T cells in B6 mice (data not shown). RAG-DIETER mice are DIETER mice bred onto the B6 RAG-deficient background. PD-1-deficient mice have been described¹⁹. All mice, including B6 mice and B6 mice carrying the allotypic marker CD90.1 (B6-Thy-1.1), were bred in the Biologisches

Zentrallabor (University of Zurich) and were used at an age of 6–10 weeks. We always used sex- and age-matched animals for adoptive transfers and for bone marrow chimeras. Animal experiments were done according to institutional guidelines and to Swiss federal and cantonal laws on animal protection.

Generation of bone marrow chimeras. Recipient mice were lethally irradiated (10 Gy) with a ^{60}Co source and were injected intravenously with 2×10^6 to 5×10^6 bone marrow cells that were collected from tibias and femurs of donor mice. Mice were given 1 mg/ml of sulfadoxin and 0.2 mg/ml of trimethoprim in the drinking water during the first 2 weeks. Chimeras were used in experiments no earlier than 6 weeks after transplantation. For the generation of 'RAG-DIETER + B6' chimeras, B6 recipients were lethally irradiated and were reconstituted with 70% RAG-DIETER plus 30% B6 bone marrow. For the generation of mixed PD-1-deficient chimeras, B6-Thy-1.1 mice were lethally irradiated and reconstituted with 70% RAG-DIETER plus 15% B6-Thy-1.1 plus 15% PD-1-deficient (Thy-1.2) bone marrow: Wild-type and knockout T cells are thus present in the same mouse and can be separately analyzed with the allotypic marker Thy-1. Before use in experiments, flow cytometry was done on blood samples to verify an equal reconstitution of the CD8⁺ T cell compartment with wild-type and knockout T cells.

Antibodies and reagents. All antibodies used for flow cytometry were obtained from PharMingen (Becton Dickinson Biosciences). The monoclonal antibody 4F10 (Armenian hamster IgG) is directed against mouse CTLA-4 (CD152)⁵². Injection of 0.5 mg or of 2 mg of 4F10 did not induce upregulation of MHC class II, CD54 or CD86 on CD11c⁺ cells *in vivo*, thus excluding the possibility that treatment of mice with 4F10 resulted in activation of DCs (data not shown). Control hamster IgG was obtained from MP Biomedicals. The following peptides were synthesized by Neosystem: GP(33–41) (KAVYNFTAC), GP(276–286) (SGVENPGGYCL), NP(396–404) (FQPQNGQFI) and β -galactosidase, amino acids 497–504 (ICPMYARV). Peptides were dissolved at a concentration of 10 mM in DMSO. Tamoxifen (ICN Biomedicals) was suspended in 96% ethanol, then 9 volumes of olive oil were added and tamoxifen was dissolved at 37 °C to obtain a solution of 20 mg/ml. LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany)⁵³ and was propagated on L929 cells at a low multiplicity of infection.

Treatment of mice. For induction of antigen presentation by resting DCs, DIETER mice or DIETER mixed bone marrow chimeras were injected intraperitoneally with 2 mg tamoxifen per 0.1 ml solvent or with 0.1 ml solvent as control. For blocking of CTLA-4 interactions, mice were given 0.5 mg 4F10, or as control, 0.5 mg hamster IgG, intraperitoneally at the same time as tamoxifen. Injection of mice with 0.5 mg 4F10 or control hamster IgG did not result in activation of DCs, as measured by flow cytometry staining for CD11c plus CD86, CD11c plus CD54, and CD11c plus MHC class II (data not shown). A commonly used control for putative nonspecific effects of 4F10 is commercially available polyclonal hamster IgG, which is derived from Syrian hamsters, whereas 4F10 was generated from Armenian hamsters. We therefore used mice treated with tamoxifen rather than tamoxifen plus hamster IgG as controls in some experiments (Figs. 4 and 5). We nevertheless included hamster IgG as a control in another experiment (Fig. 6). For LCMV infection, 100 PFU LCMV-WE was given intravenously.

For adoptive transfer of naive splenocytes, single-cell suspensions equivalent to one spleen (2×10^7 to 4×10^7 cells) of naive, allotypically different, but age- and sex-matched mice were adoptively transferred into DIETER bone marrow chimeras by intravenously injection. The time point of adoptive transfer relative to tamoxifen injection is stated for each experiment.

Staining with tetrameric MHC class I–peptide complexes. Tetrameric complexes containing biotinylated H-2D^b or H-2K^b, β_2 -microglobulin, the relevant peptide and extravidin-phycoerythrin were generated, and staining was done as described^{54,55}. Blood or spleen cells were stained for 10 min at 37 °C in flow cytometry buffer (PBS containing 2% FCS, 0.03% NaN₃ and 2mM EDTA) with 1 μg tetramer, followed by staining with anti-CD8 β -fluorescein isothiocyanate (clone 53-6.7) and anti-Thy-1.2-biotin (clone 53-2.1) followed by staining with streptavidin-allophycocyanin. Tetramer-positive cells were determined after gating on live CD8⁺, allotypic marker-positive or allotypic marker-negative cells.

Intracellular staining for IFN- γ . Approximately 1×10^6 splenocytes were incubated for 6 h at 37 °C in a total volume of 200 μl medium with 2 μM of the relevant peptide, with medium alone, or with 50 ng/ml phorbol 12-myristate 13-acetate plus 500 ng/ml of ionomycin in the presence of 5 $\mu\text{g}/\text{ml}$ of brefeldin A and 50 U/ml of recombinant mouse IL-2. After incubation, cells were surface-stained with anti-CD8 α -phycoerythrin (clone 5H10-1) and with anti-Thy-1.2-biotin (clone 53-2.1) followed by staining with streptavidin-allophycocyanin, if applicable. Cells were then fixed with 2% paraformaldehyde and were permeabilized with permeabilization buffer (flow cytometry buffer containing 0.1% saponin) followed by intracellular staining with anti-mouse IFN- γ -fluorescein isothiocyanate (clone AN18). IFN- γ ⁺ cells were determined after gating on CD8⁺, Thy-1.2⁺ or Thy-1.2⁻ cells.

Statistical analysis. Statistical analysis was done with Student's *t*-test and Prism 4 software (GraphPad Software).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Mondino, A., Khoruts, A. & Jenkins, M.K. The anatomy of T-cell activation and tolerance. *Proc. Natl. Acad. Sci. USA* **93**, 2245–2252 (1996).
- Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* **2**, 1032–1039 (2001).
- Kyewski, B., Derbinski, J., Gotter, J. & Klein, L. Promiscuous gene expression and central T-cell tolerance: more than meets the eye. *Trends Immunol.* **23**, 364–371 (2002).
- Bjorses, P., Aaltonen, J., Horelli-Kuitunen, N., Yaspo, M.L. & Peltonen, L. Gene defect behind APECED: a new clue to autoimmunity. *Hum. Mol. Genet.* **7**, 1547–1553 (1998).
- Anderson, M.S. *et al.* Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1395–1401 (2002).
- Bouneaud, C., Kourilsky, P. & Bouso, P. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity* **13**, 829–840 (2000).
- Walker, L.S. & Abbas, A.K. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol.* **2**, 11–19 (2002).
- Steinman, R.M. *et al.* Dendritic cell function *in vivo* during the steady state: a role in peripheral tolerance. *Ann. NY Acad. Sci.* **987**, 15–25 (2003).
- Steinman, R.M., Hawiger, D. & Nussenzweig, M.C. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* **21**, 685–711 (2003).
- Sakaguchi, S. Naturally arising CD4⁺ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* **22**, 531–562 (2004).
- Tivol, E.A. *et al.* Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* **3**, 541–547 (1995).
- Waterhouse, P. *et al.* Lymphoproliferative disorders with early lethality in mice deficient in Ctl4. *Science* **270**, 985–988 (1995).
- Perez, V.L. *et al.* Induction of peripheral T cell tolerance *in vivo* requires CTLA-4 engagement. *Immunity* **6**, 411–417 (1997).
- Walunas, T.L. & Bluestone, J.A. CTLA-4 regulates tolerance induction and T cell differentiation *in vivo*. *J. Immunol.* **160**, 3855–3860 (1998).
- Lindsten, T. *et al.* Characterization of CTLA-4 structure and expression on human T cells. *J. Immunol.* **151**, 3489–3499 (1993).
- Brunner, M.C. *et al.* CTLA-4-mediated inhibition of early events of T cell proliferation. *J. Immunol.* **162**, 5813–5820 (1999).
- Ishida, Y., Agata, Y., Shibahara, K. & Honjo, T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* **11**, 3887–3895 (1992).
- Agata, Y. *et al.* Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int. Immunol.* **8**, 765–772 (1996).
- Nishimura, H., Nose, M., Hiai, H., Minato, N. & Honjo, T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* **11**, 141–151 (1999).
- Nishimura, H. *et al.* Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* **291**, 319–322 (2001).

21. Freeman, G.J. *et al.* Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med.* **192**, 1027–1034 (2000).
22. Latchman, Y. *et al.* PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol.* **2**, 261–268 (2001).
23. Okazaki, T., Iwai, Y. & Honjo, T. New regulatory co-receptors: inducible co-stimulator and PD-1. *Curr. Opin. Immunol.* **14**, 779–782 (2002).
24. Chen, L. Co-inhibitory molecules of the B7–CD28 family in the control of T-cell immunity. *Nat. Rev. Immunol.* **4**, 336–347 (2004).
25. Probst, H.C., Lagnel, J., Kollias, G. & van den Broek, M. Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8⁺ T cell tolerance. *Immunity* **18**, 713–720 (2003).
26. Moser, M. Dendritic cells in immunity and tolerance-do they display opposite functions? *Immunity* **19**, 5–8 (2003).
27. Gueronprez, P., Valladeau, J., Zitvogel, L., Thery, C. & Amigorena, S. Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* **20**, 621–667 (2002).
28. Scheinecker, C., McHugh, R., Shevach, E.M. & Germain, R.N. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J. Exp. Med.* **196**, 1079–1090 (2002).
29. Belz, G.T. *et al.* The CD8 α ⁺ dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J. Exp. Med.* **196**, 1099–1104 (2002).
30. Lin, Y., Roberts, T.J., Sriram, V., Cho, S. & Brutkiewicz, R.R. Myeloid marker expression on antiviral CD8⁺ T cells following an acute virus infection. *Eur. J. Immunol.* **33**, 2736–2743 (2003).
31. Probst, H.C. *et al.* Immunodominance of an antiviral cytotoxic T cell response is shaped by the kinetics of viral protein expression. *J. Immunol.* **171**, 5415–5422 (2003).
32. Hou, W.S. & Van Parijs, L. A Bcl-2-dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells. *Nat. Immunol.* **5**, 583–589 (2004).
33. Ingulli, E., Mondino, A., Khoruts, A. & Jenkins, M.K. *In vivo* detection of dendritic cell antigen presentation to CD4⁺ T cells. *J. Exp. Med.* **185**, 2133–2141 (1997).
34. Robinson, S.P., Langan-Fahey, S.M., Johnson, D.A. & Jordan, V.C. Metabolites, pharmacodynamics, and pharmacokinetics of tamoxifen in rats and mice compared to the breast cancer patient. *Drug Metab. Dispos.* **19**, 36–43 (1991).
35. Krummel, M.F. & Allison, J.P. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J. Exp. Med.* **182**, 459–465 (1995).
36. Dhodapkar, M.V. & Steinman, R.M. Antigen-bearing immature dendritic cells induce peptide-specific CD8⁺ regulatory T cells *in vivo* in humans. *Blood* **100**, 174–177 (2002).
37. Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. & Enk, A.H. Induction of interleukin 10-producing, nonproliferating CD4⁺ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* **192**, 1213–1222 (2000).
38. Mahnke, K., Qian, Y., Knop, J. & Enk, A.H. Induction of CD4⁺/CD25⁺ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* **101**, 4862–4869 (2003).
39. Groux, H. *et al.* A transgenic model to analyze the immunoregulatory role of IL-10 secreted by antigen-presenting cells. *J. Immunol.* **162**, 1723–1729 (1999).
40. Bonifaz, L. *et al.* 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 (2002).
41. Hawiger, D. *et al.* Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. *J. Exp. Med.* **194**, 769–779 (2001).
42. Misra, N., Bayry, J., Lacroix-Desmazes, S., Kazatchkine, M.D. & Kaveri, S.V. Cutting edge: human CD4⁺CD25⁺ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J. Immunol.* **172**, 4676–4680 (2004).
43. Albert, M.L. Death-defying immunity: do apoptotic cells influence antigen processing and presentation? *Nat. Rev. Immunol.* **4**, 223–231 (2004).
44. Hernandez, J., Aung, S., Redmond, W.L. & Sherman, L.A. 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 (2001).
45. Hugues, S. *et al.* Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. *Immunity* **16**, 169–181 (2002).
46. Kurts, C., Kosaka, H., Carbone, F.R., Miller, J.F. & Heath, W.R. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8⁺ T cells. *J. Exp. Med.* **186**, 239–245 (1997).
47. Morgan, D.J., Kreuzel, H.T. & Sherman, L.A. Antigen concentration and precursor frequency determine the rate of CD8⁺ T cell tolerance to peripherally expressed antigens. *J. Immunol.* **163**, 723–727 (1999).
48. Lo, D. *et al.* Peripheral tolerance to an islet cell-specific hemagglutinin transgene affects both CD4⁺ and CD8⁺ T cells. *Eur. J. Immunol.* **22**, 1013–1022 (1992).
49. McCoy, K.D., Hermans, I.F., Fraser, J.H., Le Gros, G. & Ronchese, F. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) can regulate dendritic cell-induced activation and cytotoxicity of CD8⁺ T cells independently of CD4⁺ T cell help. *J. Exp. Med.* **189**, 1157–1162 (1999).
50. Nishimura, H., Honjo, T. & Minato, N. Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice. *J. Exp. Med.* **191**, 891–898 (2000).
51. Blank, C. *et al.* Absence of programmed death receptor 1 alters thymic development and enhances generation of CD4/CD8 double-negative TCR-transgenic T cells. *J. Immunol.* **171**, 4574–4581 (2003).
52. Walunas, T.L. *et al.* CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1**, 405–413 (1994).
53. Lehmann-Grube, F. Lymphocytic Choriomeningitis Virus. *Virology Monogr.* **10**, 1–173 (1971).
54. Gallimore, A. *et al.* Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J. Exp. Med.* **187**, 1383–1393 (1998).
55. Probst, H.C., Dumrese, T. & van den Broek, M.F. Cutting edge: competition for APC by CTLs of different specificities is not functionally important during induction of antiviral responses. *J. Immunol.* **168**, 5387–5391 (2002).
56. Battagay, M. *et al.* Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. *J. Virol. Methods* **33**, 191–198 (1991).