Innate $CD4^+CD25^+$ regulatory T cells are required for oral tolerance and inhibition of $CD8^+$ T cells mediating skin inflammation

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To elucidate the role of $CD4^+CD25^+$ regulatory T cells in oral tolerance, we used the model of contact hypersensitivity (CHS) to 2,4-dinitrofluorobenzene (DNFB), which is mediated by $CD8^+$ Tc1 effector cells independently of $CD4^+$ T-cell help. Conversely to normal mice, invariant chain knock-out (KO) ($Ii^{o/o}$) mice, which are deficient in $CD4^+$ T cells, cannot be orally tolerized and develop a chronic hapten-specific CHS response. Transfer of naive $CD4^+$ T cells before hapten gavage into $Ii^{o/o}$ mice restores oral tolerance by a mechanism independent of interleukin-10 (IL-10) production by $CD4^+$ T cells. That naturally occurring $CD4^+CD25^+$ T cells are critical for oral tolerance induction is demonstrated by the finding that (1) transfer of $CD4^+CD25^+$ but not $CD4^+CD25^-$ T cells into $Ii^{o/o}$ recipients completely prevents the CHS response and skin infiltration by $CD8^+$ T cells, by blocking development of hapten-specific $CD8^+$ T cells; (2) in vivo depletion of $CD4^+CD25^+$ cells by antibody treatment in normal mice impairs oral tolerance; and (3) $CD4^+CD25^+$ T cells inhibit hapten-specific $CD8^+$ T-cell proliferation and interferon γ (IFNγ) production, in vitro. These data show that naturally occurring $CD4^+CD25^+$ T cells are instrumental for orally induced tolerance and are key actors for the control of antigen-specific $CD8^+$ T-cell effectors mediating skin inflammation. (Blood. 2003;102:3295-3301)

Introduction

Oral tolerance has long been recognized as a physiologic mechanism of immune unresponsiveness to dietary antigens and bacterial microflora antigens, which maintain tissue integrity by preventing harmful delayed-type hypersensitivity (DTH) responses in the intestine and may also limit the efficiency of oral vaccination. Indeed, antigen encounter in the intestine triggers an active inhibitory process preventing the onset of $CD4^+$ and $CD8^+$ T-cell antigen-specific immune responses to subsequent systemic immunization with the same antigen (reviewed in Garside and Mowat[1]). Several mechanisms have been proposed to explain peripheral tolerance induced by antigen feeding. These include anergy[2,3] or deletion of antigen-specific T cells,[4,5] immune deviation to Th2-biased immune response, and induction of regulatory Th3 (transforming growth factor β [TGFβ]–producing) cells.[6,7]

The naturally occurring regulatory subset of $CD4^+CD25^+$ T cells accounting for 5% to 10% of peripheral $CD4^+$ T cells has been extensively reported to exert potent immunosuppressive function in vivo and in vitro toward $CD4^+$ T-cell effectors[8] and may represent regulatory T cells responsible for orally induced peripheral tolerance. Indeed, $CD4^+CD25^+$ T cells, which arise from the thymus as early as day 3 of life,[9] are characterized by a memory phenotype; low proliferative capacity and interleukin-2 (IL-2) production; secretion of high levels of the immunosuppressive cytokines IL-10 and TGF-β; and expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4),[10-13] a molecule that contributes to orally induced tolerance.[14] These cells have been described in a variety of experimental models to protect from autoimmune diseases, as well as colitis and allograft rejection.[8] Reminiscent to these cells, IL-10–producing ovalbumin (OVA)–specific $CD4^+$ T clones (Tr1) generated in vitro after repeated stimulation with antigen in the presence of IL-10 were shown to prevent colitis when cotransferred with naive $CD4^+CD45RB^{high}$ T cells in OVA-fed immunocompromised severe combine immunodeficiency (SCID) or Nude mice.[15] Interestingly, mice genetically deficient for either IL-2, IL-2R, T-cell receptor αβ (TcRαβ), TGFβ, IL-10, or major histocompatibility complex (MHC) class II were shown to develop spontaneous colitis,[16-20] compatible with a shared physical or functional defect in the regulatory $CD4^+CD25^+$ subset. Although recent studies in TcR transgenic models have reported that oral antigen delivery can induce activation and/or differentiation of regulatory $CD4^+CD25^+$ T cells,[21,22] evidence that they are instrumental for in vivo induction of oral tolerance has not been provided. Moreover, evidence as to whether $CD4^+CD25^+$ cells are responsible for peripheral suppression of antigen-specific $CD8^+$ cytotoxic T-lymphocyte (CTL) responses is still sparse.[23]

In this study we examined whether $CD4^+CD25^+$ T cells contribute to oral tolerance in normal nonlymphopenic hosts, using a pathophysiologic model of antigen-specific skin inflammation mediated by $CD8^+$ CTL effectors. Contact hypersensitivity (CHS) to the hapten 2,4-dinitrofluorobenzene (DNFB) provides a unique model to address this issue because (1) skin inflammation generated upon skin challenge with the DNFB in sensitized mice is

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mediated by specific MHC class I–restricted interferon γ (IFNγ)–producing $CD8^+$ CTL effectors, independently of $CD4^+$ T-cell help[24-28]; (2) feeding mice once with the hapten prior to skin sensitization completely abrogates the CHS response by blocking development of specific IFNγ-producing $CD8^+$ CTLs[29,30]; and (3) oral tolerance cannot be induced in mice deficient in $CD4^+$ T cells. We now show that $CD4^+CD25^+$ T cells are mandatory for orally induced tolerance and block in vivo development of hapten-specific $CD8^+$ T-cell–mediating skin inflammation.

Materials and methods

Mice

All mice were used at 6 to 10 weeks of age and were on a C57BL/6 background (H-2^b). Female C57BL/6 mice were purchased from Charles Rivers Laboratories (l'Arbresle, France). MHC class II ($A\beta^{\circ/\circ}$)[31] and invariant chain ($Ii^{\circ/\circ}$)[32]–deficient mice were kindly provided by D. Mathis and C. Benoist. IL-10–deficient mice were obtained from Dr W. Mueller (Institute for Genetics, University of Cologne, Germany).[20] All mice were bred as homozygotes in Charles Rivers Laboratories.

Contact sensitivity assay

CHS to DNFB was determined by the mouse ear-swelling test.[33] Briefly, mice were sensitized epicutaneously on day 0 by application of 25 μL of 0.5% 2,4-dinitrofluorobenzene (DNFB; Sigma, St Quentin Fallavier, France) diluted in acetone–olive oil (4:1, vol/vol) onto 2 cm^2 of shaved abdominal skin. Mice were challenged on day 5 with 4 μL of a nonirritant concentration of 0.25% DNFB applied onto each side of the right ear. The left ear received the vehicle alone. Ear thickness was measured using a caliper (J15 Blet; Lyon, France) before and at various times after challenge. The ear swelling (micrometers) was calculated as ($T-T_0$ of the right ear) − ($T-T_0$ of the left ear), where T_0 and T represent the values of ear thickness before and after the challenge, respectively. Ear swelling in unsensitized but ear-challenged mice was usually less than 20 μm. Statistical significance was calculated by the Mann-Whitney-Wilcoxon ranking test.

Induction of oral tolerance

Mice were orally tolerized by a single intragastric administration of 300 μL of either 0.1% DNFB in acetone–olive oil (1:10, vol/vol) or vehicle alone (as control), 7 days before sensitization with DNFB (day −7), as previously described.[29] For adoptive transfer experiments, nonfractionated $CD4^+$ T cells or purified $CD4^+$ T-cell subsets were transferred intravenously into $Ii^{\circ/\circ}$ or $A\beta^{\circ/\circ}$ recipient mice, 16 hours before feeding (day −8). For depletion experiments, mice were injected with either a control rat monoclonal antibody (mAb) or a depleting anti-CD25 mAb (clone PC61) on days −10, −7, −3, and 0.

Immunohistochemical staining of $CD8^+$ T cells

Cryostat sections of the ears were incubated for one hour with anti-CD8 rat mAb (clone 53-6.7 from Pharmingen, San Diego, CA) or an irrelevant rat mAb as control, followed by a biotinylated mouse adsorbed goat anti–rat immunoglobulin G (IgG) Ab. Specific binding was revealed with a streptavidin-peroxidase kit (Dako, Glostrup, Denmark) and AEC (3-amino-9-ethylcarbazole) as previously described.[30] Sections were counterstained with hematoxylin.

Purification of T-cell subsets

Spleens and lymph nodes (mesenteric, inguinal, and axillary) were used to prepare single cell suspensions. For most experiments, $CD4^+$ and $CD8^+$ T cells were isolated by positive selection using anti-CD4– or anti-CD8–coated microbeads and selection columns (Miltenyi Biotec, Bergish Gladbach, Germany). Purity was routinely more than 95%. For certain experiments, $CD4^+$ and $CD8^+$ T cells were enriched by negative selection using columns coated with a goat anti–mouse Ig, a goat anti–rat IgG, and a rat antimouse CD4 or CD8 mAbs (Biotex, Edmonton, AB, Canada). In this case, a purity of more than 80% was routinely obtained. $CD4^-$ cells were purified using LD depletion columns (Miltenyi Biotec).

For isolation of $CD4^+$ T-cell subsets, $CD4^+$ T cells were first enriched from spleen, mesenteric lymph nodes (MLNs), and peripheral lymph node cell suspensions by negative selection using anti–MHC class II, anti-CD11b, and anti-CD8 mAbs and magnetic beads. Enriched $CD4^+$ T cells were then incubated with biotin-conjugated anti-CD25 mAb (7D4) and PE–anti-CD4 followed by fluorescein isothiocyanate (FITC)–conjugated streptavidin. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were then purified by flow cytometry using a FACStar cell sorter (BD Biosciences, San Jose, CA). Alternatively, for most in vivo transfer experiments, cell suspensions were sequentially incubated with biotin-conjugated anti-CD25 mAb (7D4) (15μg/10^8 cells) (Pharmingen), FITC-conjugated streptavidin, and anti-FITC microbeads (Miltenyi Biotec). $CD4^+CD25^+$ cells were isolated by 2 runs on LS selection columns and were routinely more than 90% pure. For isolation of $CD4^+CD25^-$ cells, stained suspensions were first depleted of $CD25^+$ cells using depletion columns, and $CD4^+$ T cells were purified using anti-CD4 microbeads and positive selection columns. $CD4^+CD25^-$ cells were always more than 90% $CD4^+$ and less than 1% $CD25^+$.

Preparation of APCs

Bone marrow–derived dendritic cells (BM-DCs) and syngeneic naive spleen cells were used as antigen-presenting cells (APCs) for in vitro experiments. DCs were generated from bone marrow cells as previously described,[34] with some modifications. In brief, bone marrow was flushed from tibias and femurs prior to red blood cell depletion. Cells were cultured at 37°C in 24-well culture (2×10^5/mL/well) in complete RPMI medium supplemented with 40 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ). Half of the medium was renewed every other 2 days by fresh medium and GM-CSF. Cells were collected after 7 days and were routinely more than 70% $CD11c^+$. Spleen cells or BM-DCs were first incubated for 20 minutes at 37°C with 4 mM 2,4-dinitrobenzenesulfonate (DNBS) or medium alone (serum-free RPMI) and washed 3 times in complete medium. Hapten-pulsed APCs were then treated with mitomycin C (25 μg/mL) for 25 minutes at 37°C in complete medium and thoroughly washed before use.

Hapten-specific $CD8^+$ T-cell proliferation and IFNγ production

$CD8^+$ T cells were isolated from spleen and abdominal skin draining lymph nodes from mice 5 days after DNFB skin sensitization. $CD8^+$ cells ($1\text{-}2 \times 10^5$) were cultured in round-bottom 96-well plates in the presence of DNBS-pulsed mitomycin C–treated splenocytes (APCs/CD8 = 2.5:1) or BM-DCs (APCs/CD8 = 1:5). $CD4^+CD25^+$ cells were purified from naive or orally tolerized mice and added at various numbers into cultures. The proliferative response was assessed after 3 days of culture by [^{3}H]thymidine incorporation (1 μCi/well [0.037 MBq/well]) during the last 8 hours. The cultures were harvested and the amount of [^{3}H]thymidine uptake was counted using a β-plate liquid scintillation counter. The results are expressed as Δcpm ± SD, where Δcpm = (cpm in cultures of T cells with DNBS-pulsed APCs)−(cpm in cultures of T cells with untreated APCs). Cell-free supernatants were harvested after 48 hours, and IFNγ production was titrated by enzyme-linked immunosorbent assay (ELISA) using rat antimouse γ-IFN clone R46-A2 as the capture mAb and biotin-conjugated rat antimouse γ-IFN clone XMG1.2 as secondary mAb (both from Pharmingen).

Results

Experimental model

We have previously reported oral tolerance breakdown in 3 distinct experimental models of $CD4^+$ T-cell deficiency: (1) anti-CD4 mAb–treated C57BL/6 mice (with a complete defect in $CD4^+$ T cells), (2) MHC class II knock-out ($A\beta^{\circ/\circ}$) mice (with complete

defect in MHC class II–restricted CD4+ T cells), and (3) invariant-chain–deficient (Ii°/°) mice (with partial defect in MHC class II–restricted CD4+ T cells). We previously reported that conversely to normal C57BL/6 mice, in which a single oral administration of the hapten (DNFB) can block development of the CHS response and of hapten-specific CD8+ effector cells, mice deficient in CD4+ T cells in each of these models are refractory to tolerance induction,[29,30] suggesting a role for regulatory CD4+ T cells in oral tolerance. In this study we used Ii°/° mice, which have residual MHC class II–expressing APCs[32] and develop preferential Th1 immune response[35] as well as strong and chronic CHS to DNFB,[29] as a model to investigate the ability of various CD4+ T-cell subsets to restore oral tolerance.

CD4+ T cells can restore oral tolerance

As previously observed,[29] oral administration of DNFB prior to skin sensitization in Ii°/° mice failed to induce tolerance and affected neither the magnitude nor the kinetics of the skin inflammatory response to hapten challenge (Figure 1A). Adoptive transfer before hapten gavage of CD4+ T cells (but not CD4-depleted cells) from naive C57BL/6 mice restored oral tolerance in Ii°/° mice and prevented skin inflammation (Figure 1A). This effect was correlated with the number of CD4+ T cells transferred, inasmuch as 10^6 CD4+ T cells were inefficient, while 9×10^6 CD4+ T cells induced up to 80% inhibition of the CHS response (Figure 1B). Transfer of CD4+ T cells in control vehicle–fed skin-sensitized Ii°/° mice did not affect the CHS response (data not shown). Thus, CD4+ T cells and orally administered hapten are both required to block the onset of hapten-induced skin inflammation in invariant chain–deficient mice.

Contribution of MHC class II molecules and IL-10 in restoration of oral tolerance by CD4+ T cells

Ii°/° mice have residual MHC class II molecules, which might be required for oral tolerance induction through activation of CD4+ regulatory cells, expressed on the cell surface of APCs.[32] To address this hypothesis, we performed adoptive transfer experiments in MHC class II–deficient (Aβ°/°) recipient mice. As shown in Figure 2A and in contrast to Ii°/° mice, MHC class II–deficient mice were not tolerized when naive CD4+ T cells were adoptively transferred prior to hapten feeding. Thus, CD4+ T cells required the presence of host MHC class II–positive APCs in order to restore oral tolerance.

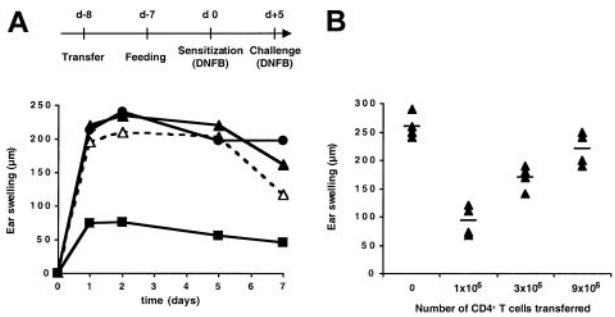

Figure 1. Adoptive transfer of CD4+ T cells restored oral tolerance in invariant chain– deficient mice . Ii°/° mice were either left untreated or transferred intravenously on day −8 with (A) 10×10^6 CD4+ T cells or CD4-depleted cells from naive C57BL/6 mice or (B) graded numbers of CD4+ T cells. All mice were fed either vehicle or DNFB one day later, sensitized epicutaneously with 0.5% DNFB on day 0, and ear challenged with 0.25% DNFB on day +5. The CHS response was determined by ear swelling at various times (A) or 48 hours (B) after hapten challenge. Standard errors were less than 15% (A). Mean increases in ear thickness are indicated by horizontal bars (B). In panel A, △ indicates untreated mice fed with vehicle; ▲, untreated mice fed with DNFB; ■, mice transferred with CD4+ T cells and fed with DNFB; and ●, mice transferred with CD4-depleted cells and fed with DNFB.

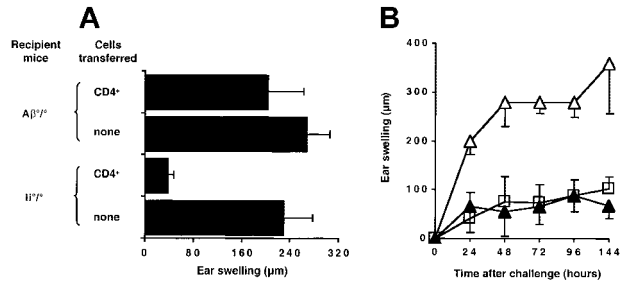

Figure 2. Restoration of oral tolerance by CD4+ T cells is MHC class II–dependent but is not mediated by IL-10 secretion. (A) Naive CD4+ T cells (10×10^6) were transferred intravenously into Ii°/° or Aβ°/° recipient mice on day −8. (B) CD4+ T cells (10×10^6) purified from either naive wild-type C57BL/6 (▲) or IL-10–deficient (□) mice were adoptively transferred in Ii°/° mice on day −8. Groups of mice without cell transfer (△) were used as control. All mice were then fed DNFB on day −7, sensitized on day 0 with DNFB, and ear challenged on day +5. Ear swelling was determined at 48 hours (A) or at various times after challenge (B). Results are expressed as mean values ± SD and are representative of 3 independent experiments.

Oral tolerance of the CHS response to DNFB is greatly impaired both in IL-10°/° mice and in normal mice treated with neutralizing anti–IL-10 antibody (data not shown). To examine whether IL-10 produced by CD4+ T cells contributed to oral tolerance induction, we compared the ability of CD4+ T cells from either control or IL-10–deficient mice to restore susceptibility to oral tolerance upon transfer into Ii°/° recipient mice. As depicted in Figure 2B, CD4+ T cells from either naive IL-10°/° or C57BL/6 mice transferred one day before feeding were equally efficient at restoring oral tolerance in Ii°/° recipient mice, indicating that IL-10 production by CD4+ T cells was not involved in their regulatory function in vivo.

In vivo depletion of CD25+ T cells by antibody treatment abrogates oral tolerance

To address the role of CD4+CD25+ cells in oral tolerance, normal C57BL/6 mice were treated with a depleting anti-CD25 mAb (PC61) or a rat IgG mAb as control and tested for oral tolerance induction. Flow cytometry analysis using an anti-CD4 mAb and the anti-CD25 clone 7D4 (directed against an epitope of the molecule distinct from that recognized by PC61) confirmed depletion of more than 95% of CD4+CD25+ cells from both blood and spleen (data not shown). In hapten-fed mice, depletion of CD25+ cells resulted in the abrogation of tolerance as indicated by the appearance of a significant CHS response after skin sensitization (Figure 3). These data suggest that naturally occurring CD4+CD25+ T cells represent a critical regulatory cell subset during oral tolerance induction in vivo.

CD4+ CD25+ T cells restore oral tolerance in Ii°/° mice by preventing hapten-specific skin inflammation

To further demonstrate that CD4+CD25+ T cells are required for oral tolerance induction, we examined whether CD4+CD25+ T-cell transfer could prevent CHS only when transferred before DNFB gavage in Ii°/° mice. As shown in Figure 4A, CD4+CD25+ T cells were unable to suppress CHS in vehicle-fed Ii°/° recipients but achieved complete suppression of CHS in DNFB-fed mice. This confirms that concomitant hapten feeding is mandatory for the ability of CD4+CD25+ T-cell transfer to restore tolerance. To determine whether CD4+CD25+ T cells represented the major regulatory subset able to restore oral tolerance, we compared the

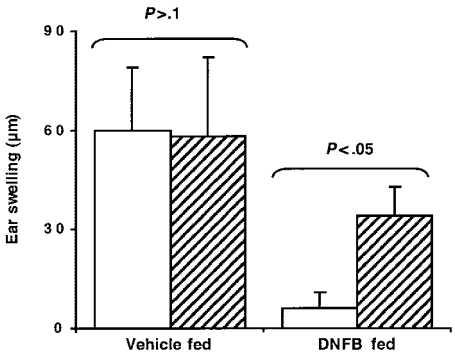

Figure 3. Anti-CD25 mAb treatment impairs oral tolerance in normal mice. C57BL/6 mice were injected intraperitoneally with either a control rat mAb (□) or a depleting anti-CD25 mAb (▨) on days −10, −7, −3, and 0, with respect to day 0 of DNFB sensitization as illustrated in Figure 1. Mice were fed either vehicle or DNFB, sensitized, and ear challenged with DNFB. Ear-swelling responses were determined at 48 hours after challenge.

capacity of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells to restore oral tolerance in $Ii^{\circ/\circ}$ mice. To this end, both subsets were purified from naive C57BL/6 mice by cell sorting and transferred before gavage into $Ii^{\circ/\circ}$ mice, in cell number representing the relative proportion of each subset among total unfractionated $CD4^+$ T cells. As shown in Figure 4B, as few as 1×10^6 $CD4^+CD25^+$ cells prevented the development of the skin inflammatory response as efficiently as 10×10^6 total $CD4^+$ T cells. In contrast, transfer of 9×10^6 $CD4^+CD25^-$ cells resulted in only partial and transient down-regulation of the CHS. These results demonstrate that only $CD4^+CD25^+$ T cells allowed maximal and stable restoration of oral tolerance in $Ii^{\circ/\circ}$ mice.

We have previously reported that skin inflammation in CHS is mediated by hapten-specific cytolytic $CD8^+$ T cells recruited at the site of hapten challenge[27,28] and that lack of CHS in orally tolerized mice correlated with absence of $CD8^+$ T cells in the challenged skin.[30] Immunohistochemical analysis was thus carried out to examine the relative outcome of $CD4^+$ T-cell subset transfer on skin inflammation and infiltration with $CD8^+$ T cells upon challenge in the same $Ii^{\circ/\circ}$ recipients as those shown in Figure 4. As expected, untransferred $Ii^{\circ/\circ}$ mice had an intense skin inflammatory reaction manifested by dermal edema and fibrosis associated with a massive cellular infiltration of both dermis and epidermis (Figure 5A) containing many $CD8^+$ T cells (Figure 5E). Similar skin inflammatory infiltrate and $CD8^+$ T-cell recruitment (Figure 5D,H) occurred in $Ii^{\circ/\circ}$ recipients of $CD4^+CD25^-$ T cells. In contrast, $Ii^{\circ/\circ}$ mice tolerized by transfer of either $CD4^+$ T cells (Figure 5B,F) or $CD4^+CD25^+$ T cells (Figure 5C,G) exhibited a normal skin histology with no sign of inflammation and complete lack of $CD8^+$ T cells. Thus, the efficacy of $CD4^+CD25^+$ but not $CD4^+CD25^-$ cells to restore oral tolerance and prevent CHS correlated with lack of $CD8^+$ T-cell effectors recruited in the skin.

$CD4^+$ $CD25^+$ cells tolerize hapten-specific $CD8^+$ T cells in vivo

Because $CD4^+CD25^+$ T-cell–mediated restoration of oral tolerance in $Ii^{\circ/\circ}$ mice could result from impaired priming/expansion of hapten-specific $CD8^+$ effector cells, as observed in orally tolerized normal mice,[30] we next analyzed the presence of hapten-specific $CD8^+$ T cells in secondary lymphoid organs of hapten-fed $Ii^{\circ/\circ}$ recipients on day 5 after skin sensitization. As expected, hapten feeding in $Ii^{\circ/\circ}$ mice prior to skin sensitization was unable to inhibit the development of hapten-specific $CD8^+$ effector T-cell response in secondary lymphoid organs (Figure 6). Likewise, adoptive transfer of the $CD4^+CD25^-$ T-cell subset did not affect the hapten-specific $CD8^+$ T-cell response. Alternatively, adoptive transfer of either 10×10^6 unfractionated $CD4^+$ T cells or 10^6 $CD4^+CD25^+$ T cells completely prevented hapten-specific $CD8^+$ T-cell proliferation within secondary lymphoid organs (Figure 6). Altogether, these data demonstrated that $CD4^+CD25^+$ T cells restored oral tolerance in Ii-deficient mice by preventing the priming/expansion of hapten-specific $CD8^+$ CHS effector T cells.

$CD4^+$ $CD25^+$ T cells inhibit hapten-specific $CD8^+$ T-cell responses in vitro

We next examined whether $CD4^+CD25^+$ T cells could directly inhibit hapten-specific $CD8^+$ T-cell responses and whether hapten feeding potentiated their suppressive function. For this purpose, hapten-primed $CD8^+$ T cells were purified from spleen and draining lymph nodes of DNFB-sensitized mice and were restimulated in vitro with hapten-pulsed bone marrow–derived DCs in the presence of graded numbers of $CD4^+CD25^+$ T cells purified from either naive or tolerant mice. $CD4^+CD25^+$ T cells from naive or tolerant mice were hyporesponsive to in vitro stimulation and did not proliferate in response to hapten-pulsed DCs (data not shown). As shown in Figure 7, $CD4^+CD25^+$ T cells from both naive and tolerant mice suppressed in a dose-dependent manner $CD8^+$ T-cell proliferation and γ-IFN production, both resulting in more than 70% inhibition when used at a 1:1 ratio with effector $CD8^+$ T cells. $CD4^+CD25^+$ T cells isolated from tolerant mice were reproducibly found to have slightly higher suppressive activity, compared with $CD4^+CD25^+$ cells isolated from naive donors, especially when used at lower cell ratios. Thus, naturally occurring $CD4^+CD25^+$ cells can control hapten-specific $CD8^+$ T-cell responses, and oral exposure to antigen enhanced their suppressive function.

Discussion

This study demonstrates that $CD4^+$ T cells are MHC class II–dependent regulatory cells responsible for orally induced tolerance of $CD8^+$ T-cell–mediated CHS responses and that $CD4^+CD25^+$ cells represent the major subset responsible for this effect. Remarkably, adoptive transfer of naive $CD4^+$ T cells can restore complete oral tolerance in otherwise refractory invariant chain–deficient mice. $CD4^+$ T-cell transfer in the absence of hapten feeding failed to inhibit the CHS response, indicating that $CD4^+$ T cells and oral hapten were both required to achieve oral tolerization.

We showed that within $CD4^+$ T lymphocytes, the naturally occurring $CD4^+CD25^+$ T-cell subset is responsible for restoration

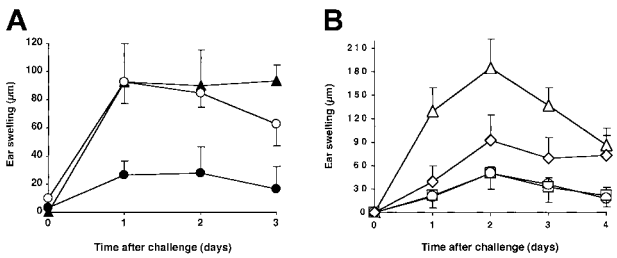

Figure 4. $CD4^+$ $CD25^+$ regulatory cells restore oral tolerance in $Ii^{\circ/\circ}$ mice . (A) $Ii^{\circ/\circ}$ mice were left untreated (▲) or transferred intravenously with 1.10^6 naive $CD4^+CD25^+$ T cells one day before gavage (ie, day −8) with either DNFB (●) or vehicle alone (○). (B) $Ii^{\circ/\circ}$ mice were left untreated (△) or transferred intravenously with either naive total $CD4^+$ T cells (10×10^6; □), $CD4^+CD25^-$ T cells (9×10^6; ◇), or $CD4^+CD25^+$ T cells (1×10^6; ○) one day before gavage with DNFB. All mice were sensitized epicutaneously on day 0, and the CHS response was measured as described in Figure 1 legend. The data are expressed as mean values ± SD and are representative of at least 3 independent experiments.

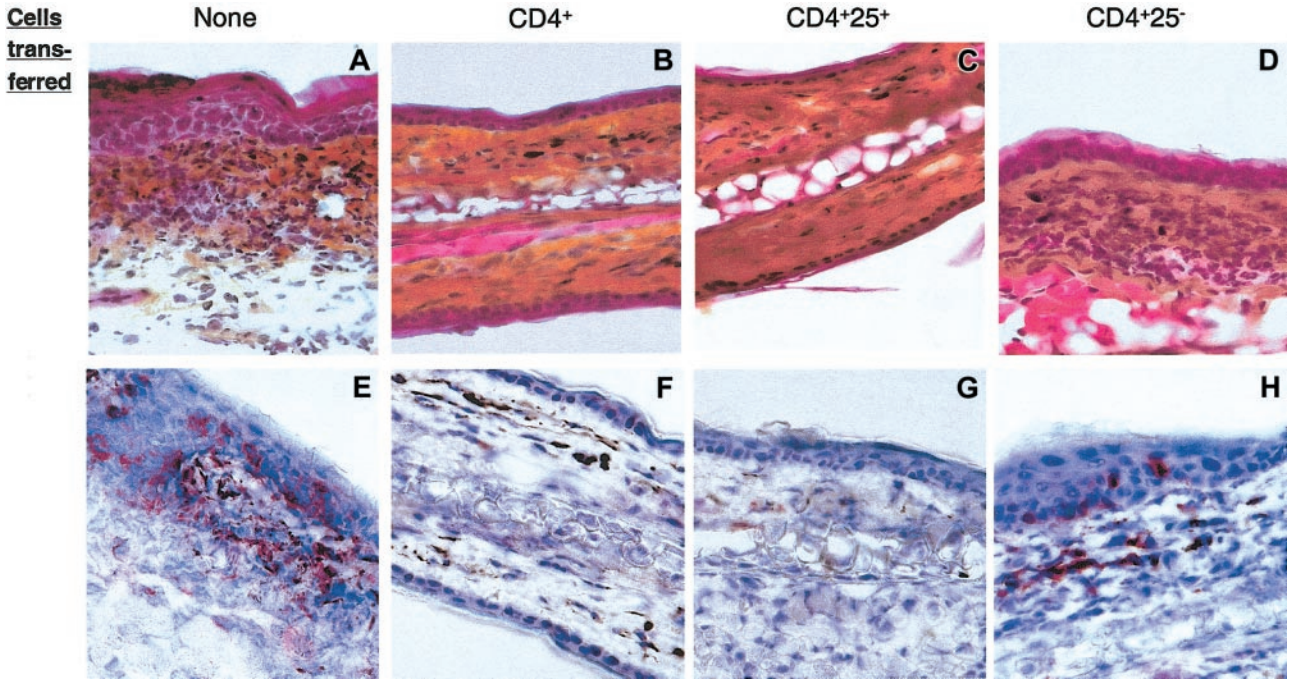

Figure 5. CD4+ CD25+ T cells prevent CD8+-mediated skin inflammation. Ears from the same Ii$^{\circ/\circ}$ recipients as those in Figure 3 were harvested 96 hours after DNFB challenge. Cryostat sections of ears were either stained with hematoxylin/phloxin/safran (A-D) or with an anti-CD8 mAb (red) and counter-stained with hematoxylin (E-G). Original magnification, × 40. No CD8-specific staining or local inflammatory reaction was detected in sections of ears from nonsensitized animals (not shown).

of oral tolerance. Indeed, in vivo transfer of as few as 10^6 purified $CD4^+CD25^+$ T cells to Ii$^{\circ/\circ}$ recipient mice before hapten feeding completely prevented the CHS response induced by subsequent skin sensitization, while 10.10^6 $CD4^+$ T cells were required to achieve similar suppression. The suppressive effect of transferred $CD4^+CD25^+$ T cells on the CHS response required concomitant antigen feeding, since $CD4^+CD25^+$ cell transfer did not affect the CHS response of vehicle-fed Ii$^{\circ/\circ}$ recipients. That $CD4^+CD25^+$ but not $CD4^+CD25^-$ T cells were responsible for the in vivo regulatory effect of unfractionated $CD4^+$ T cells was confirmed by the finding that only $CD4^+CD25^+$ T-cell transfer was able to prevent (1) the development and differentiation of hapten-specific $CD8^+$ T cells in secondary lymphoid organs and (2) $CD8^+$ T-cell infiltration in the hapten-challenged skin of the Ii$^{\circ/\circ}$ recipient. In addition, in vivo depletion of $CD25^+$ T cells by specific antibody treatment impaired oral tolerance in normal C57BL/6 mice, although not completely as compared with anti-CD4 mAb depletion.[30] This could be explained by either (1) incomplete depletion of $CD4^+CD25^+$ cells, especially from tissues such as the intestine, (2) concomitant depletion of activated $CD8^+$ effectors that have up-regulated CD25, or (3) the ability of $CD4^+CD25^-$ cells to exert some level of regulation, as reported in other models.[21,36-38] Indeed, $CD4^+CD25^-$ cells were found to inhibit wasting or autoimmune disease in lymphopenic host to the same extent as $CD4^+CD25^+$ cells.[36,37] In addition, TcR transgenic $CD4^+CD25^-$ T cells were reported to differentiate in vivo into $CD4^+CD25^+$ regulatory T cells upon activation with antigen expressed in peripheral tissues[38] or encountered following oral delivery.[21] Although transfer of $CD4^+CD25^-$ T cells induced a partial and transient decrease in the CHS response in some mice, these cells were unable to prevent skin infiltration by $CD8^+$ T cells or development of the hapten-specific $CD8^+$ T-cell response in secondary lymphoid organs. It has recently been emphasized that the relative homeostatic expansion capacity of T-cell subsets transferred in immunocompromised SCID or RAG$^{\circ/\circ}$ (recombination activating genes) hosts plays an important role in their

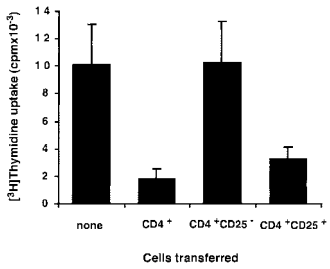

Figure 6. CD4+ CD25+ cells prevent expansion of hapten-specific CD8+ T cells. Ii$^{\circ/\circ}$ mice were either left untreated or were transferred intravenously on day −8 with either naive total $CD4^+$ T cells (10×10^6), $CD4^+CD25^-$ T cells (9×10^6), or $CD4^+CD25^+$ T cells (1×10^6), fed with DNFB on day −7 and skin sensitized on day 0 with DNFB. Purified $CD8^+$ T cells (2×10^5) from spleen and lymph nodes, harvested on day 5 after sensitization, were restimulated in vitro for 3 days with syngeneic mitomycin C–treated spleen cells (5×10^5) either untreated or pulsed with DNBS. T-cell proliferation was determined by [3H]thymidine uptake during the last 8 hours. Results are expressed as Δcpm values (ie, cpm from hapten-derivatized spleen cells − cpm from untreated spleen cells) ± SD of triplicate wells.

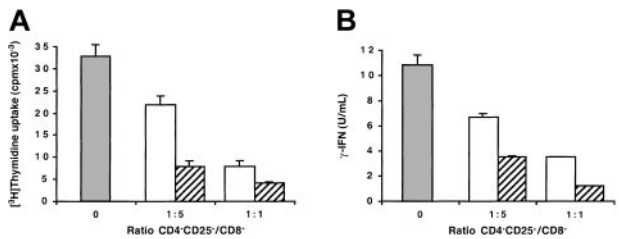

Figure 7. Innate CD4+ CD25+ cells inhibit hapten-specific CD8+ expansion in vitro. $CD8^+$ T cells (10^5) were isolated from day-5 DNFB-sensitized C57BL/6 mice and stimulated with hapten-pulsed and mitomycin C–treated BM-DCs (2×10^4) (▨). Graded numbers of $CD4^+CD25^+$ T cells, purified from spleen and lymph nodes of either naive (□) or orally tolerant C57BL/6 mice (▨), were added to cultures. Proliferation was determined after 3 days by [3H]thymidine uptake (A) and γ-IFN production was titrated by ELISA in 48 hours cell free supernatants (B). Results are expressed as mean values ± SD and are representative of 3 independent experiments.

suppressive properties.[39] Although Ii$^{\circ/\circ}$ mice are not immunocompromised, it is possible that the partial and transient protection induced by CD4$^+$CD25$^-$ cells might relate to the large number of cells transferred and/or to the homeostatic expansion that may occur to some extent, resulting in competition with hapten-specific CD8$^+$ effector T cells.

Our finding that naive regulatory CD4$^+$CD25$^+$ cells could block in vivo development and/or differentiation of antigen-specific CD8$^+$ T-cell effectors during orally induced tolerance confirms and extends to a pathophysiologic situation, previous data showing that CD8$^+$ T cells can be targets of regulatory CD4$^+$CD25$^+$ T cells in vitro, via direct T-T interaction.[40] This raises the question of the mechanism(s) by which regulatory CD4$^+$CD25$^+$ T cells prevent CD8$^+$ T-cell expansion during oral tolerance and control CD8$^+$ T-cell–mediated inflammatory responses in Ii$^{\circ/\circ}$ mice. It is possible that hapten penetration through the gut mucosa activates, or favors the differentiation of, a pool of regulatory CD4$^+$CD25$^+$ T cells that recirculate via blood or lymph and are readily present in skin draining lymph nodes at the time of skin sensitization, thus preventing priming/expansion of hapten-specific CD8$^+$ effector cells during the afferent phase of the CHS response. Alternatively, hapten feeding may prime hapten-specific CD8$^+$ T cells, which are inactivated or deleted by regulatory CD4$^+$CD25$^+$ T cells, thus resulting in lack of functional hapten-specific CD8$^+$ T cells available at the time of skin sensitization. This latter hypothesis is supported by studies reporting that orally induced systemic tolerance to protein antigens is preceded by rapid activation of specific CD8$^+$ CTLs in Peyer patches and mesenteric lymph nodes[41,42] and by our finding that CD4$^+$CD25$^+$ T cells can block IFNγ production and proliferation of hapten-primed CD8$^+$ T cells upon in vitro restimulation with the hapten.

It may be proposed that MHC class II–dependent interaction between CD4$^+$CD25$^+$ T cells and the APCs that have captured the hapten from the gut is required to trigger their suppressive function. Indeed, the inability of CD4$^+$CD25$^+$ T cells to restore oral tolerance in Aβ$^{\circ/\circ}$ recipients indicates that CD4$^+$CD25$^+$ cells need MHC class II molecule expression by host APCs to exert their regulatory function and is reminiscent of a recent study showing that peripheral MHC class II molecules allow maintenance of regulatory CD4$^+$CD25$^+$ T cells in lymphopenic hosts.[43] In addition, CD4$^+$CD25$^+$ T cells harvested from orally tolerized normal mice exhibited a more potent regulatory effect on hapten-specific CD8$^+$ T-cell responses in vitro, suggesting that hapten feeding could affect the size, regulatory activity, or migratory capacity of CD4$^+$CD25$^+$ T cells. In this respect, recent studies reported that oral antigen administration in recipients receiving TcR transgenic T cells could increase the size of the regulatory CD4$^+$CD25$^+$ T-cell pool at the periphery.[21,22]

Whether regulatory T cells, and CD4$^+$CD25$^+$ T cells in particular, exert peripheral suppression via a bystander or an antigen-specific mechanism is still debated. Numerous studies have described oral tolerance as an antigen-specific mechanism, because systemic immune response to a nominal antigen (either a protein or a hapten) could be prevented only by prior oral administration of the same antigen. Likewise, our previous studies of oral tolerance in the model of CHS showed that even when mice were double sensitized with 2 non–cross-reacting haptens (ie, DNFB and OXA) to generate effector cells specific for both, tolerance was induced exclusively by feeding with the same hapten as the one used for challenge.[30] It is possible that hapten feeding activates and/or expands antigen-specific CD4$^+$CD25$^+$ T cells recognizing complexes of MHC class II/hapten-modified peptides. Alternatively, the apparent antigen specificity of T-cell regulation may relate to the fact that the APCs may simultaneously present the oral hapten to CD8$^+$ T cells and activate regulatory CD4$^+$CD25$^+$ T cells via self-peptide/MHC class II complexes. Such bridging could allow tolerization of hapten-specific CD8$^+$ T cells by naturally occurring CD4$^+$CD25$^+$ T cells.

Whether IL-10 plays a role in oral tolerance and whether it is responsible for the regulatory function of CD4$^+$CD25$^+$ T cells are of major importance. This latter issue has yielded divergent results that may be related to differences in the experimental systems used. Thus, IL-10 production appears crucial for the ability of CD4$^+$CD25$^+$ cells to prevent colitis in immunocompromised mice[44] but not for their inhibitory effect on gastritis.[45] Furthermore, IL-10 production by CD4$^+$CD25$^+$ cells was shown to be mandatory for controlling the inflammatory response induced by bacterial superantigen in CD25-deficient mice.[46] In our model, IL-10 production by CD4$^+$CD25$^+$ T cells did not account for induction of CD8$^+$ T-cell tolerance. Indeed, (1) CD4$^+$ T cells from IL-10–deficient mice were as efficient as wild-type CD4$^+$ T cells to restore oral tolerance to CHS in Ii$^{\circ/\circ}$ mice, (2) hapten feeding in normal mice did not potentiate IL-10 production by CD4$^+$CD25$^+$ T cells, and (3) neutralizing anti–IL-10 or anti–IL-10 receptor mAbs did not affect their ability to inhibit the hapten-specific CD8$^+$ T-cell response in vitro (data not shown). Nevertheless, we found that IL-10 is critical for efficient induction of oral tolerance, inasmuch as oral tolerance of the CHS response to DNFB is greatly impaired both in IL-10$^{\circ/\circ}$ mice as well as in normal mice treated with neutralizing anti–IL-10 antibody (B.D., D.K., unpublished observations, 2003). Thus, IL-10, which is constitutively produced by intestinal epithelial cells[47] and Peyer patch dendritic cells,[48] may be a critical factor in the gut microenvironment at the time of antigen penetration for efficient tolerization of CD8$^+$ T cells. IL-10 might be also instrumental for regulatory T-cell differentiation, as demonstrated for Tr1 cells,[15,49] and by limiting local activation of hapten-specific CD8$^+$ T cells, may render them more susceptible to CD4$^+$CD25$^+$ T-cell regulation.

This study documents the potent in vivo regulatory effect of CD4$^+$CD25$^+$ T cells on orally induced tolerance of CD8$^+$ T cells mediating antigen-specific tissue inflammation. Successful therapy by oral antigen and CD4$^+$CD25$^+$ cells in Ii$^{\circ/\circ}$ mice, who have Th1-biased T-cell responses, supports the potential clinical and therapeutic interest of CD4$^+$CD25$^+$ cells for the control of CD8$^+$ Tc1-mediated diseases.

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