Innate CD4⁺CD25⁺ regulatory T cells are required for oral tolerance and control CD8⁺ T cells mediating skin inflammation

Short title: CD4⁺CD25⁺ regulatory T cells and oral tolerance

Bertrand Dubois¹, Ludivine Chapat¹, Anne Goubier¹, Martine Papiernik²
Jean-François Nicolas³ and Dominique Kaiserlian¹

¹INSERM U404 "Immunité et Vaccination", IFR 128 Bioscience Lyon Gerland; Tour INSERM - 21, avenue Tony Garnier, 69365 Lyon cedex 07 France
²INSERM U591, CHU Necker-enfants malades, 156 rue de vaugirard 75730 Paris Cedex 15 France
³INSERM U503 "Immunobiologie Fondamentale et Clinique", IFR 128 Bioscience Lyon Gerland; Tour INSERM - 21, avenue Tony Garnier, 69365 Lyon cedex 07 France

Address correspondence to D.Kaiserlian:

INSERM U404 – IFR 128 - 21, avenue Tony Garnier 69365 LYON cedex 07 – France
phone: (33) 4 37 28 23 94 - fax: (33) 4 37 28 23 91
kaiserlian@cervi-lyon.inserm.fr

Financial supports:

This work was supported by an institutional grant from Institut National de la Santé et de la Recherche Médicale and a specific grant from Association pour la Recherche sur le Cancer (n° 4296 to D.K.).

Word counts:

Abstract: 200 - Text: 4826

Scientific heading: Immunobiology

Abbreviations:

CHS : contact hypersensitivity, DNFB: 2,4-dinitrofluorobenzene, Ii: invariant chain,
ABSTRACT

To elucidate the role of CD4+CD25+ regulatory T cells in oral tolerance, we used the model of contact hypersensitivity (CHS) to DNFB, which is mediated by CD8+ Tc1 effector cells independently of CD4+ T cell help. Conversely to normal mice, invariant chain KO (Ii-/-) mice, which are deficient in CD4+ T cells, cannot be orally tolerized and develop a chronic hapten-specific CHS response. Transfer of naïve CD4+ T cells before hapten gavage into Ii-/- mice restores oral tolerance by a mechanism independent of 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: (i) transfer of CD4+CD25+ but not CD4+CD25- T cells into Ii-/- recipients completely prevents the CHS response and skin infiltration by CD8+ T cells, by blocking development of hapten-specific CD8+ T cells, (ii) in vivo depletion of CD4+CD25+ cells by antibody treatment in normal mice impairs oral tolerance and (iii) CD4+CD25+ T cells inhibit hapten-specific CD8+ T cell proliferation and 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.

kaiserlian@cervi-lyon.inserm.fr
INTRODUCTION

Oral tolerance has long been recognized as a physiological mechanism of immune unresponsiveness to dietary antigens and bacterial microflora antigens, which maintains tissue integrity by preventing harmful 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 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 (TGFβ-producing) cells 6,7.

The naturally occurring regulatory subset of CD4+CD25+ T cells accounting for 5-10% of peripheral CD4+ T cells, has been extensively reported to exert potent immuno-suppressive 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 IL-2 production, secretion of high levels of the immunosuppressive cytokines IL-10 and TGF-β and expression of CTLA-4 10-13, a molecule which contributes to orally-induced tolerance 14. These cells have been described in a variety of experimental models to protect from auto-immune diseases, as well as colitis and allograft rejection 8. Reminiscent to these cells, IL-10-producing-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 co-transferred with naive CD4+CD45RBhigh T cells in OVA-fed immuno-compromised SCID or Nude mice 15. Interestingly, mice genetically deficient for either IL-2, IL-2R, TcRαβ, TGFβ, IL-10 or 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, whether CD4⁺CD25⁺ cells are responsible for peripheral suppression of antigen-specific CD8⁺ CTL responses is still sparse ²³.

In this study we examined whether CD4⁺CD25⁺ T cells contribute to oral tolerance in normal non-lymphopenic hosts, using a patho-physiological model of antigen-specific skin inflammation mediated by CD8⁺ CTL effectors. Contact hypersensitivity (CHS) to the hapten DNFB provides a unique model to address this issue, because (i) skin inflammation generated upon skin challenge with the DNFB in sensitized mice is mediated by specific MHC class I-restricted IFNγ-producing CD8⁺ CTL effectors, independently of CD4⁺ T cell help ²⁴-²⁸, (ii) feeding mice with the hapten once prior to skin sensitization completely abrogates the CS response by blocking development of specific IFNγ-producing CD8⁺ CTL ²⁹,³⁰ and (iii) 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 wk 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^°/°) \(^{31}\) and invariant chain (Ii°/°) \(^{32}\) 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

CS 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, v/v) onto 2 cm\(^2\) of shaved abdominal skin. Mice were challenged on day 5 with 4 µl of a non irritant 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 time 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 un-sensitized but ear challenged mice was usually <20 µm. Statistical significance were calculated by the Mann-Witney-Wilcoxon Ranking test.

Induction of oral tolerance

Mice were orally tolerized by a single intra-gastric administration of 300 µl of either 0.1 % DNFB in acetone-olive oil (1:10, v/v) or vehicle alone (as control), seven days before sensitization with DNFB (day -7), as previously described \(^{29}\). For adoptive transfer experiments, non-fractionated CD4\(^+\) T cells or purified CD4\(^+\) T cell subsets were transferred iv into Ii°/° or Aβ°/° recipient mice, 16 hours before feeding (day –8). For depletion experiments, mice were injected with either a control rat mAb or a depleting anti-CD25 mAb (Clone PC61) on day-10, -7, -3 and 0.
Immunohistochemical staining of CD8+ T cells

Cryostat sections of the ears were incubated for 1 hour with anti-CD8 rat mAb (clone 53-6.7 from Pharmingen) or an irrelevant rat mAb as control, followed by a biotinylated mouse adsorbed goat anti-rat IgG Ab. Specific binding was revealed with a streptavidin-peroxidase kit (Dako, Glostrup, Denmark) and AEC as previously described. 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 micro-beads and selection columns (Miltenyi Biotec, Bergish Gladbach, Germany). Purity was routinely >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 anti-mouse CD4 or CD8 mAbs (Biotex, Edmonton, Canada). In this case, a purity >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, MLN and peripheral lymph nodes cell suspensions by negative selection using anti-MCH-II, -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 FITC-conjugated streptavidin. CD4+CD25+ and CD4+CD25− cells were then purified by flow cytometry using a FACStar cell sorter (BD Biosciences). Alternatively, for most in vivo transfer experiments cell suspensions were sequentially incubated with biotin-conjugated anti-CD25 mAb (7D4) (15µg/10^8 cells) (Pharmingen, San Diego, CA), FITC-conjugated streptavidin and anti-FITC micro-beads (Miltenyi Biotec). CD4+CD25+ were isolated by two runs on LS selection columns and were routinely >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 micro-beads and positive selection columns. CD4+CD25- cells were always >90% CD4+ and <1% CD25+.

**Preparation of APCs**

Bone marrow derived DC (BM-DC) and syngeneic naive spleen cells were used as APCs for *in vitro* experiments. DC 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 cells depletion. Cells were cultured at 37°C in 24-well culture (2x10^5/ml/well) in complete RPMI medium supplemented with 40 ng/ml of recombinant murine GM-CSF (Peprotech, France). Half of the medium was renewed every other two day by fresh medium and GM-CSF. Cells were collected after 7 days and were routinely >70% CD11c+. Spleen cells or BM-DC were first incubated for 20 min at 37°C with 4mM 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 min 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 five days after DNFB skin sensitization. CD8+ cells (1-2x10^5) were cultured in round bottom 96 wells plates in the presence of DNBS-pulsed Mitomycin C treated splenocytes (APCs/CD8 = 2.5:1) or BM-DC (APCs/CD8=1:5). CD4+CD25+ 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 [3H]thymidine incorporation (1 µCi/well) during the last 8 hours. The cultures were harvested and the amount of [3H]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 γ-IFN production was titrated by ELISA using rat anti-mouse γ-IFN.
clone R46-A2 as the capture mAb and biotin-conjugated rat anti-mouse \( \gamma \)-IFN clone XMG1.2 as secondary mAb (both from Pharmingen).

RESULTS

Experimental model

We have previously reported oral tolerance breakdown in three distinct experimental models of CD4\(^+\) T cell deficiency: (i) anti-CD4 mAb treated C57BL/6 mice (with a complete defect in CD4\(^+\) T cells), (ii) MHC-class-II knock-out (A\(^{\beta -}\)) mice (with complete defect in MHC class II-restricted CD4\(^+\) T cells) and (iii) Invariant-chain-deficient (I\(^{\text{ii}}\)\(^{-/}\)) 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 I\(^{\text{ii}}\)\(^{-/}\) mice, which have residual MHC class II expressing APC \(^{32}\) 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 I\(^{\text{ii}}\)\(^{-/}\) mice, failed to induce tolerance and affected neither the magnitude nor the kinetics of the skin inflammatory response to hapten challenge (Fig. 1A). Adoptive transfer before hapten gavage of CD4\(^+\) T cells (but not CD4-depleted cells) from naive C57BL/6 mice restored oral tolerance in I\(^{\text{ii}}\)\(^{-/}\) mice and prevented skin inflammation (Fig. 1A). This effect was correlated with the number of CD4\(^+\) T cells transferred, inasmuch as 10\(^6\) CD4\(^+\) T cells were inefficient while 9x10\(^6\) CD4\(^+\) T cells induced up to 80% inhibition of the CHS response (Fig. 1B). Transfer of CD4\(^+\) T cells in control vehicle-fed skin sensitized I\(^{\text{ii}}\)\(^{-/}\) 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 expressed on the cell surface of APCs, which might be required for oral tolerance induction through activation of CD4$^+$ regulatory cells. To address this hypothesis, we performed adoptive transfer experiments in MHC-class-II deficient (Aβ$^{−/−}$) recipient mice. As shown in Fig. 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.

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 Fig. 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.
A

Recipient mice

Cells transferred

Aβ*↓

CD4⁺
none

B

Cells transferred

Eur swelling (µm)

Time after challenge (hours)

CD4⁺

CD4⁺-IL10

CD4⁺-IL10

Dubois et al. Fig. 2
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% 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 (Fig. 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^<sup>−/−</sup> 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^<sup>−/−</sup> mice. As shown in Fig 4A, CD4^+CD25^+ T cells were unable to suppress CHS in vehicle-fed Ii^<sup>−/−</sup> recipient, 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 capacity of CD4^+CD25^+ and CD4^+CD25^- T cells to restore oral tolerance in Ii^<sup>−/−</sup> mice. To this end, both subsets were purified from naive C57BL/6 mice by cell sorting and transferred before gavage into Ii^<sup>−/−</sup> mice, in cell number representing the relative proportion of each subset among total un-fractinated CD4^+ T cells. As shown in Fig. 4B, as few as 1x10^6 CD4^+CD25^+cells prevented the development of the skin inflammatory response as efficiently as 10x10^6 total CD4^+ T cells. In contrast transfer of 9x10^6 CD4^+CD25^- cells only resulted in 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^<sup>−/−</sup> 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 subsets transfer on skin inflammation and infiltration with CD8$^+$ T cells upon challenge in the same Ii$^{−/−}$ recipients as those shown in Fig. 4. As expected, un-transferred Ii$^{−/−}$ mice had an intense skin inflammatory reaction manifested by dermal oedema and fibrosis associated with a massive cellular infiltration of both dermis and epidermis (fig. 5A) containing many CD8$^+$ T cells (fig. 5E). Similar skin inflammatory infiltrate and CD8$^+$ T cells recruitment (Fig. 5D and H) occurred in Ii$^{−/−}$ recipients of CD4$^+$CD25$^-$ T cells. In contrast, Ii$^{−/−}$ mice tolerized by transfer of either CD4$^+$ T cells (fig 5B and 5F) or CD4$^+$CD25$^+$ T cells (fig. 5C and 5G) 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+ tolerize hapten-specific CD8+ T cells in vivo

Because CD4+CD25+ T cell mediated restoration of oral tolerance in Il−/− 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 Il−/− recipients on day 5 after skin sensitization. As expected, hapten feeding in Il−/− mice prior to skin sensitization was unable to inhibit the
development of hapten-specific CD8\(^+\) effector T cell response in secondary lymphoid organs (Fig. 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 10x10\(^6\) un-fractionnated CD4\(^+\) T cells or 10\(^6\) CD4\(^+\)CD25\(^+\) T cells completely prevented hapten-specific CD8\(^+\) T cell proliferation within secondary lymphoid organs (Fig. 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.
Dubois et al. Fig. 6
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 re-stimulated in vitro with hapten-pulsed bone marrow-derived DC 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 hypo-responsive to in vitro stimulation and did not proliferate in response to hapten-pulsed DC (data not shown). As shown in Fig. 7, CD4+CD25+ T from both naive and tolerant mice suppressed in a dose dependent manner CD8+ T cell proliferation and γ-IFN production, both resulting in >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, as compared to CD4+CD25+ cells isolated from naive donors, especially when used at lower cell ratios. Thus, naturally occurring CD4+CD25+ can control hapten specific CD8+ T cell responses, and oral exposure to antigen enhanced their suppressive function.
A

$[^3]H\text{-Tdr}$ uptake (c.p.m.$\times 10^2$)

Ratio CD4+CD25+CD8+

B

IFN (U/ml)

Ratio CD4+CD25+CD8+

Dubois et al. Fig. 7
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+ represent the major subset responsible for this effect. Remarkably, adoptive transfer of naive CD4+ T cell 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 of oral tolerance. Indeed, in vivo transfer of as few as 10^6 purified CD4+CD25+ T cells to Ii^−/− 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 cell on the CHS response required concomitant antigen feeding, since CD4+CD25+ cell transfer did not affect the CHS response of vehicle-fed Ii^−/− recipients. That CD4+CD25+ but not CD4+CD25− T cells were responsible for the in vivo regulatory effect of un-fractionated CD4+ T cells was confirmed by the finding that only CD4+CD25+ T cell transfer was able to prevent (i) the development and differentiation of hapten-specific CD8+ T cells in secondary lymphoid organs and (ii) CD8+ T cell infiltration in the hapten-challenged skin of Ii^−/− 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 to anti-CD4 mAb depletion 30. This could be explained by either (i) incomplete depletion of CD4+CD25+ cells, especially from tissues such as the intestine, (ii) concomitant depletion of activated CD8+ effectors that have up-regulated CD25, or (iii) the ability of CD4+CD25− cells to exert some level of regulation, as reported in other models 21,36-38. Indeed, CD4+CD25− were found to inhibit wasting or auto-immune disease in lymphopenic host to the same extent as CD4+CD25+. 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 nor 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 immuno-compromised SCID or RAG\(^{\gamma\delta}\) hosts plays an important role in their suppressive properties \(^{39}\). Although Ii\(^{\gamma\delta}\) mice are not immuno-compromised, it is possible that the partial and transient protection induced by CD4\(^+\)CD25\(^-\) cells might relate to the large number of cell transferred and/or to the homeostatic expansion that may occur to some extend, 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 patho-physiological 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\(^{\gamma\delta}\) 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 re-circulate 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 preceeded by rapid activation of specific CD8\(^+\) CTL in Peyer’s 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 APC 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 Ab⁺⁻ recipients indicates that CD4⁺CD25⁺ cells need MHC-class II molecules expression by host APC to exert their regulatory function and is reminiscent to a recent study showing that peripheral MHC class II molecules allow maintenance of regulatory CD4⁺CD25⁺ T cells in lymphopenic hosts. 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 recipient receiving TcR transgenic T cells could increase the size of the regulatory CD4⁺CD25⁺ T cell pool at the periphery.

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 two non cross reacting haptens (i.e. 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. 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 APC 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 is of major importance. This latter issue has yielded divergent results which may be related to differences in the experimental systems used. Thus IL-10 production appears crucial for the ability of CD4⁺CD25⁺ to prevent colitis in immuno-compromised 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 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, (i) CD4⁺ T cells from IL-10 deficient mice were as efficient as wild type CD4⁺ T cells to restore oral tolerance to CHS in Il⁻/⁻ mice, (ii) hapten feeding in normal mice did not potentiate IL-10 production by CD4⁺CD25⁺ T cells and (iii) 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⁻/⁻ mice as well as in normal mice treated with neutralizing anti-IL-10 antibody (unpublished observations). Thus, IL-10, which is constitutively produced by intestinal epithelial cells ⁴⁷ and Peyer’s 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 Il⁻/⁻ 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.
AKNOWLEDGMENTS:

We thank Grégoire Joubert for expert technical assistance with immunohistochemistry and M. Chevalier for HPS stainings.
FIGURES LEGENDS

Figure 1: Adoptive transfer of CD4+ T cells restored oral tolerance in invariant chain deficient mice

Ii°/° mice were either left un-treated or transferred iv on day-8 with (A) 10x10^6 CD4+ T cells or CD4-depleted cells from naive C57BL/6 mice or (B) graded numbers of CD4+ T cells. One day later all mice were fed either vehicle or DNFB, sensitized epicutaneously with 0.5% DNFB on day0 and ear challenged with 0.25% DNFB on day+5. The CHS response was determined by ear swelling at various time (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.

Figure 2: Restoration of oral tolerance by CD4+ T cells is MHC class-II dependent but is not mediated by IL-10 secretion

(A) 10x10^6 naive CD4+ T cells were transferred iv into Ii°/° or Aβ°/° recipient mice on day-8. (B) 10x10^6 CD4+ T cells 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 day0 with DNFB and ear challenged on day+5. Ear swelling was determined at 48 hours (A) or at various time after challenge (B). Results are representative of three independent experiments.

Figure 3: Anti-CD25 mAb treatment impairs oral tolerance in normal mice

C57BL/6 mice were injected i.p. with either a control rat mAb (white bars) or a depleting anti-CD25 mAb (shaded bars) on days-10, -7, -3 and 0, with respect to day 0 of DNFB sensitization as illustrated in Fig. 1. Mice were either fed vehicle (left panel) or DNFB (right panel), sensitized and ear challenged with DNFB. Ear swelling responses were determined at 48 hours after challenge.

Figure 4: CD4+CD25+ regulatory cells restore oral tolerance in Ii°/° mice

(A): Ii°/° mice were left untreated or transferred iv with 1.10^6 naive CD4+CD25+ T cells one day before gavage (i.e. day –8) with either DNFB or vehicle alone. B): Ii°/° mice were left untreated
or transferred iv with either naive total CD4+ T cells (10x10^6), CD4+CD25- (9x10^6) or CD4+CD25+ T cells (1x10^6) one day before gavage with DNFB. All mice were sensitized epicutaneously on day 0 and the CHS response was measured as described in Fig 1 legend. The data are representative of at least 3 independent experiments.

**Figure 5: CD4+CD25+ T cells prevent CD8+ mediated skin inflammation**

Ears from the same Ii°/° recipients as those in Fig. 3 were harvested 96 hours post-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 x40. No CD8-specific staining nor local inflammatory reaction was detected in sections of ears from non-sensitized animals (not shown).

**Figure 6: CD4+CD25+ cells prevent expansion of hapten-specific CD8+ T cells**

Ii°/° mice were either left untreated or were transferred iv on day-8 with either naive total CD4+ T cells (10x10^6), CD4+CD25- (9x10^6) or CD4+CD25+ T cells (1x10^6), fed with DNFB on day-7 and skin sensitized on day0 with DNFB. Purified CD8+ T cells (2x10^5) from spleen and lymph nodes, harvested on day5 after sensitization, were re-stimulated in vitro for 3 days with syngeneic mitomycin-C treated spleen cells (5x10^5) either untreated or pulsed with DNBS. T cell proliferation was determined by [3H]thymidine uptake during the last 8 hours. Results are expressed as ∆cpm values (i.e., 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-DC (2x10^5) (gray histograms). Graded numbers of CD4+CD25+ T cells, purified from spleen and lymph nodes of either naive (open bars) or orally tolerant C57BL/6 mice (shaded bars), 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 hour cell free supernatants (B). Results are representative of three independent experiments
REFERENCES


24. Bour H, Peyron E, Gaucherand M, Garrigue JL, Desvignes C, Kaiserlian D, Revillard JP, Nicolas JF. Major histocompatibility complex class I-restricted CD8+ T cells and class II-


36. Stephens LA, Mason D. CD25 is a marker for CD4+ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both CD25+ and CD25- subpopulations. J Immunol. 2000;165:3105-3110.


Innate CD4+CD25+ regulatory T cells are required for oral tolerance and control CD8+ T cells mediating skin inflammation

Bertrand Dubois, Ludivine Chapat, Anne Goubier, Martine Papiernik, Jean-François Nicolas and Dominique Kaiserlian