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## **CD8<sup>+</sup>CD28<sup>-</sup> regulatory T-lymphocytes prevent experimental inflammatory bowel disease in mice**

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Short title: CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells prevent colitis

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Abbreviations: IBD, inflammatory Bowel Disease; CFSE, 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester; dnTβRII, dominant negative TGF-β receptor II; IEL, Intra Epithelial Lymphocytes; LAP, Latency Associated Peptide; LPL, Lamina Propria Lymphocytes

## **Abstract**

**Background & Aims:** Immune responses to innocuous intestinal antigens appear tightly controlled by regulatory T-lymphocytes. While CD4<sup>+</sup> T-lymphocytes have recently attracted most attention, CD8<sup>+</sup> regulatory T-cell populations are also thought to play an important role in control of mucosal immunity. However, CD8<sup>+</sup> regulatory T-cell function has mainly been studied *in vitro* and no direct *in vivo* evidence exists that they can control mucosal immune responses. We investigated the capacity of CD8<sup>+</sup>CD28<sup>-</sup> T-cells to prevent experimental inflammatory bowel disease in mice. **Methods:** CD8<sup>+</sup>CD28<sup>-</sup> regulatory T-cells were isolated from unmanipulated mice and tested for their capacity to inhibit T-cell activation in allogeneic mixed lymphocyte cultures *in vitro* and to prevent inflammatory bowel disease induced by injection of CD4<sup>+</sup>CD45RB<sup>high</sup> cells into syngeneic immunodeficient Recombinase Activating Gene-2 mutant mice. **Results:** CD8<sup>+</sup>CD28<sup>-</sup> T-lymphocytes inhibited proliferation and IFN- $\gamma$  production by CD4<sup>+</sup> responder T-cells *in vitro*. CD8<sup>+</sup>CD28<sup>-</sup> regulatory T-cells freshly isolated from spleen or gut efficiently prevented inflammatory bowel disease induced by transfer of colitogenic T-cells into immunodeficient hosts. Regulatory CD8<sup>+</sup>CD28<sup>-</sup> T-cells incapable of producing IL-10 did not prevent colitis. Moreover, inflammatory bowel disease induced with colitogenic T-cells incapable of responding to TGF- $\beta$  could not be prevented with CD8<sup>+</sup>CD28<sup>-</sup> regulatory T-cells. CD8<sup>+</sup>CD28<sup>+</sup> T-cells did not inhibit *in vitro* or *in vivo* immune responses. **Conclusions:** Our findings show that naturally occurring CD8<sup>+</sup>CD28<sup>-</sup> regulatory T-lymphocytes can prevent experimental inflammatory bowel disease in mice and suggest that these cells may play an important role in control of mucosal immunity.

## ***Introduction***

During development of T and B lymphocytes in primary lymphoid organs the genes encoding their antigen-receptors undergo random somatic rearrangements. The resulting, still immature repertoire is therefore very large and contains many cells specific for self-antigens. Probably the majority of these potentially self-reactive cells are negatively selected by induction of anergy or apoptosis<sup>1,2</sup>. However, a significant number of potentially self-reactive lymphocytes leave the primary lymphoid organs and are kept under control by “peripheral tolerance mechanisms”<sup>3</sup>. Probably the most important of these mechanisms is assured by regulatory T lymphocytes capable of suppressing adaptive and also innate immune-responses<sup>4,5</sup>. Regulatory T cells are known to control immune responses to self-antigens (e.g. those leading to autoimmune disease or eliminating transformed cells<sup>4,6</sup>) but also to nonself-antigens (e.g. during pregnancy or upon infection<sup>7,8</sup>). These cells are also known to control immune responses to innocuous (probably non-self) antigens in intestinal mucosa and, in experimental animal models, their absence can lead to inflammatory bowel disease (IBD)<sup>9</sup>. Moreover, patients with IBD appear to have defects in lamina propria regulatory T cell function<sup>10</sup>.

A large number of murine models for IBD have been developed allowing for a dissection of cellular and molecular mechanisms involved in this disease<sup>11</sup>. In the most extensively used experimental model, IBD is induced by injection of naïve (CD4<sup>+</sup>CD45RB<sup>high</sup>) T cells into syngeneic immunodeficient (e.g. Severe Combined ImmunoDeficient or Recombinase Activating Gene (RAG)-deficient) mice<sup>9</sup>. Three weeks post-transfer characteristic signs of IBD start to appear: weight-loss, diarrhea, and prostrated posture of the mice. Histological analysis of the colon usually shows significant polymorphonuclear and mononuclear cell-infiltration and hyperplasia of mucosa, severe elongation of crypts, and disappearance of

goblet cells. IFN- $\gamma$  production by colitogenic T cells has been shown to play a crucial role in this animal model for IBD<sup>12</sup>.

IBD induced by injection of CD4<sup>+</sup>CD45RB<sup>high</sup> cells into immunodeficient mice can be prevented by injection of naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T lymphocytes<sup>9</sup>. CD4<sup>+</sup>CD25<sup>+</sup> T cells from IL-10 deficient mice do not prevent colitis, demonstrating the non-redundant role of this anti-inflammatory cytokine in prevention of IBD<sup>13</sup>. Moreover, colitis induced with T cells expressing a transgenic dominant negative form of the TGF- $\beta$  receptor II (dnT $\beta$ RII), and therefore incapable of responding to TGF- $\beta$ , cannot be prevented with CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, indicating a crucial role for TGF- $\beta$ <sup>14</sup>. Another CD4<sup>+</sup> regulatory T cell population capable of preventing IBD in mice has also been described<sup>15</sup>. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells have also been found in human intestines<sup>16</sup>. Combined, these data suggest that CD4<sup>+</sup> regulatory T cells may play an important role in prevention of IBD.

Whereas the best characterized regulatory T cells are of CD4<sup>+</sup>CD25<sup>+</sup> phenotype, T lymphocytes with immunosuppressive potential have also been identified in the CD8<sup>+</sup> population. Repeated *in vitro* stimulation of human peripheral blood lymphocytes with allogeneic antigen-presenting cells gradually leads to a loss of proliferative capacity. This phenomenon is caused by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T lymphocytes<sup>17</sup>. In the mouse, CD8<sup>+</sup>CD28<sup>-</sup> cells have been shown to reduce severity of experimental autoimmune encephalomyelitis<sup>18</sup>. CD8<sup>+</sup> T cells with immunosuppressive capacity also appear to play a role in oral tolerance<sup>19</sup>. Another CD8<sup>+</sup> regulatory T cell population in the mouse is characterized by high-level expression of CD122, the IL-2 receptor  $\beta$ -chain<sup>20,21</sup>. Other naturally occurring and experimentally induced murine and human CD8<sup>+</sup> regulatory T cell populations have also been described<sup>22-27</sup>. Therefore, several naturally occurring as well as induced immunoregulatory CD8<sup>+</sup> T cell populations have been identified. However, only limited data is available on the capacity of CD8<sup>+</sup> regulatory T cells to inhibit immune responses *in vivo*.

CD8<sup>+</sup> regulatory T cell populations are also thought to be involved in control of mucosal immune responses. Human CD8<sup>+</sup> T cells with *in vitro* regulatory capacity have been shown to proliferate in cultures with intestinal epithelial cells<sup>28</sup>. Importantly, lamina propria-derived CD8<sup>+</sup> T cells from normal individuals, but not from patients affected with IBD, have *in vitro* suppressive activity<sup>10</sup>. Whereas these data strongly suggest a crucial role for regulatory CD8<sup>+</sup> T cells in mucosal tolerance, direct evidence that these cells can control IBD (e.g. in animal models) has not yet been reported.

We have analyzed the capacity of naïve CD8<sup>+</sup>CD28<sup>-</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T lymphocytes, freshly isolated from unmanipulated mice, to inhibit proliferation and IFN- $\gamma$  production by CD4<sup>+</sup> responder T cells in allogeneic mixed lymphocyte cultures. We also evaluated if naïve CD8<sup>+</sup>CD28<sup>-</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T lymphocytes can prevent experimental IBD in mice and assessed regulatory effector mechanisms employed.

## **Material and Methods**

### **Mice**

All mice (females) were used at 6 to 10 weeks of age, except where indicated differently. C57BL/6 and DBA/2 mice were purchased from Janvier (Le Genest St Isle, France). RAG-2 deficient and MHC-deficient (IA $\beta$  $\beta$ 2m $^{\circ}$ ) C57BL/6 mice were bred in our SPF animal facility and were originally obtained from the CDTA CNRS (Orléans, France). IL-10 deficient C57BL/6 mice were purchased from Charles-River (L'Arbresle, France). dnT $\beta$ RII-transgenic C57BL/6 mice<sup>29</sup> were obtained from Dr. Fiona Powrie, Oxford, UK, and maintained in the animal facility of the “Institut de Pharmacologie et de Biologie Structurale”, Toulouse, France. For *in vivo* studies with cells derived from these mice, 4 week-old animals were used. The health status of mice in the animal facility was periodically monitored according to FELASA guidelines<sup>30</sup> and generally found free of monitored pathogens. Occasionally, *Trichomonas sp.* or (unidentified) *Helicobacter sp.* (but never *H. hepaticus*) were found.

### **Isolation of IEL (Intra Epithelial Lymphocytes) and LPL (Lamina Propria Lymphocytes)**

Isolation of IEL and LPL was performed as described previously<sup>31</sup>. In brief, colon specimens were washed extensively in HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Invitrogen), opened longitudinally, and cut in pieces of 5mm. Fragments were incubated for 15 min at 37°C with stirring in HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> supplemented with 1mM DTT (Sigma, Aldrich). The tissue was then washed in HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> twice for 45 min in HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> containing 0.75 mM EDTA (Invitrogen) at 37°C with stirring. The supernatant (released IEL) was collected and washed in medium. For the isolation of LPL, fragments were washed

for 20 min in RPMI 1640 (Invitrogen) supplemented with 10% FCS, 10 mM Hepes, 2 mM L-glutamine, penicillin, streptomycin, 50  $\mu$ M 2-mercaptoethanol, 1 mM non essential amino acids, 1 mM sodium pyruvate and incubated twice 2 h in complete RPMI 1640 supplemented with 0.05 mg/mL collagenase (Sigma). The supernatant (released LPL) was collected and washed in medium.

### *Flow cytometry analysis*

The following reagents were purchased from eBiosciences (San Diego, CA): FITC-conjugated Ab specific for CD44 (IM7), CD8 (53.6.7), IFN- $\gamma$  (XMG1.2) and CD45RB (IM7); PE-conjugated anti-CD28 (37.51); APC-conjugated anti-CD4 (GK1.5), anti-CD8 (53.6.7), anti-CD25 (PC61), and anti-IL-10 (JES5-16E3); biotin-conjugated anti-CD28 (375.1), anti-CD122 (5H4), anti-CD62L (MEL-14) and anti-Thy1.1 (HIS51); streptavidin-PE and streptavidin-PE-Cy5.5. The following reagents were purchased from BD Pharmingen (Heidelberg, Germany): APC-Cy7-conjugated Ab specific for CD8 (53.6.7) and Pacific Blue-conjugated anti-CD4 (RM4-5). Anti-human LAP (27232) was purchased from R&D Sciences (Minneapolis, MI), and biotin-labeled anti-mouse IgG1 from Southern Biotech (Birmingham, AL).

For FACS analysis, cells were incubated with antibodies in staining buffer (PBS and 2.5% FCS) for 20min and then washed. Intracellular IFN- $\gamma$  and IL-10 staining was performed as described below. Labeled cells were analyzed on a FACSCalibur using CellQuest software (BD Biosciences, San Diego, CA), or on an LSR II (BD) using Diva (BD) and FlowJo software (Tree Star, Ashland, OR).

### *Purification of T-cell subsets*

CD28<sup>-</sup> and CD28<sup>+</sup> CD8<sup>+</sup> cells were isolated as follows. Erythrocyte-depleted splenocytes were incubated with a cocktail of the following rat monoclonal antibodies (mAb): anti-FcγRII/III (2.4G2), anti-CD4 (GK1.5), and anti-MHC class II (M5). Thus labeled cells were eliminated using Dynabeads coated with sheep anti-rat IgG antibody (DynaL Biotech, Oslo, Norway). The resulting population was incubated with FITC-labeled anti-CD8 and biotinylated anti-CD28, followed by StrA-PE, and CD8<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>-</sup> cells were electronically sorted using a Coulter Epics Altra (Beckman Coulter, Fullerton, CA). Alternatively, the resulting population was incubated with biotinylated anti-CD28 and FITC-labeled anti-CD8 (53.6.7), washed, incubated with StrA-PE, washed, and thus PE-labeled CD28<sup>+</sup> cells magnetically depleted using anti-PE labeled microbeads (Miltenyi, Bergisch-Gladbach, Germany). Resulting CD28<sup>-</sup> cells were enriched in CD8<sup>+</sup> cells by incubation with anti-FITC labeled microbeads and subsequent magnetic positive selection (Miltenyi). Thus, a purity of > 93% was routinely obtained.

CD4<sup>+</sup> T cells used in *in vitro* assays were enriched from erythrocyte-depleted splenocytes by Dynabead-mediated depletion of FcγRIII<sup>+</sup>, MHC class II<sup>+</sup>, and CD8<sup>+</sup> cells, as described above.

CD4<sup>+</sup>CD45RB<sup>high</sup> T cells used to induce colitis were obtained as follows: ACK-treated splenocytes were depleted of CD8<sup>+</sup>, MHC class II<sup>+</sup>, and FcγRIII<sup>+</sup> cells as described above, CD4<sup>+</sup> cells enriched by incubation with anti-CD4-PE followed by magnetic sorting using anti-PE-labeled microbeads (Miltenyi), cells incubated with anti-CD45RB-FITC, and CD4<sup>+</sup>CD45RB<sup>high</sup> T cells electronically sorted using a Coulter Epics Altra (Beckman Coulter).

### *In vitro proliferation assays*

CD4<sup>+</sup> responder (10<sup>5</sup>) and CD8<sup>+</sup>CD28<sup>-</sup> regulatory (or CD8<sup>+</sup>CD28<sup>+</sup> control) cells (10<sup>5</sup>) were cultured in presence of APC (5x10<sup>5</sup>) in triplicate in 96-well round-bottom plates for 96 h and 1μCi of <sup>3</sup>H thymidine was added to the cultures for the last 16h. Thymidine incorporation was assessed using a Direct Beta Counter MATRIX 9600 (Packard, Downers Grove, IL).

Alternatively, T cell division *in vitro* was assessed by flow cytofluorography of 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) labeled cells. Isolated wt or dnTβRII transgenic CD4<sup>+</sup> effector cells were stained *in vitro* with the cytoplasmic dye CFSE (Sigma Aldrich) by incubating them for 10 min at 37°C with 5μM CFSE. The reaction was quenched by washing in ice-cold RPMI supplemented with 10% FCS. CFSE-labeled responders (10<sup>5</sup>) were cultured with isolated CD8<sup>+</sup>CD28<sup>-</sup> regulatory cells (10<sup>5</sup>) in presence of MHC-deficient APC (5x10<sup>5</sup>) and 0.5μg/ml anti-CD3ε antibody 2C11. After three days of culture, proliferation of CD4<sup>+</sup> responder cells was assessed by FACS gating on CD4-APC<sup>+</sup> responders.

### *Intracellular IFN-γ and IL-10 detection*

Cells from indicated cultures were re-stimulated with PMA (50ng/mL) and ionomycin (1μg/mL)(both from Sigma) for 4h at 37°C, Brefeldin A was added during the last 2h (10μg/mL Sigma). Cells were subsequently stained for indicated surface-markers, fixed with 2% paraformaldehyde for 15min at 4°C, permeabilized with 0.5% saponin, 1% BSA in PBS for 30min at RT, and finally incubated for 30min at RT with FITC-conjugated anti IFN-γ or APC-conjugated anti IL-10 in permeabilization buffer.

### *Induction and clinical and histological assessment of colitis*

C57BL/6 RAG-2<sup>-/-</sup> mice were injected i.v with  $4 \times 10^5$  syngeneic wt or dnT $\beta$ RII-transgenic CD4<sup>+</sup>CD45RB<sup>high</sup> T cells either alone or with  $2 \times 10^5$  syngeneic wt or IL-10 deficient CD8<sup>+</sup>CD28<sup>-</sup> or CD8<sup>+</sup>CD28<sup>+</sup> cells, isolated as described above.

T cell-reconstituted RAG-2 deficient mice were weighed weekly and euthanized after six weeks. A 1cm piece of the distal colon was removed and fixed in 10% buffered formol. Paraffin-embedded sections (5 $\mu$ m) were cut and stained with hematoxylin and eosin and used for microscopic assessment of colitis. Colons were graded semiquantitatively “no”, “minor”, “moderate”, or “severe” colitis in a blinded fashion. Minor colitis: minimal scattered mucosal inflammatory cell infiltrates with or without minimal epithelial hyperplasia. Moderate colitis: mild to moderate scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with mild epithelial hyperplasia and mild mucin depletion from goblet cells. Severe colitis: marked inflammatory cell infiltrates that were often transmural and associated with severe ulceration, marked epithelial hyperplasia and mucin depletion, and loss of intestinal glands.

## Results

### *Phenotypic analysis of CD8<sup>+</sup>CD28<sup>-</sup> T lymphocytes*

To assess the relation of CD8<sup>+</sup>CD28<sup>-</sup> T cells to other previously reported CD8<sup>+</sup> regulatory T lymphocytes, we analyzed the phenotype of these cells by flow-cytometry (Fig. 1). C57BL/6 splenocytes were stained with antibodies specific for CD4, CD8, and CD28 or an isotype-matched control antibody (Fig. 1a). CD8<sup>+</sup> T cells generally expressed slightly lower levels of CD28 than CD4<sup>+</sup> cells. However, no clear CD8<sup>+</sup>CD28<sup>-</sup> population could be distinguished. CD8<sup>+</sup>CD28<sup>-</sup> cells were therefore defined as those expressing CD28 at background levels. The thus defined CD28<sup>-</sup> population represented 26±3% of CD8<sup>+</sup> splenocytes. In two previous publications, CD122<sup>+</sup> CD8<sup>+</sup> T cells were shown to have suppressive activity<sup>20,21</sup>. We therefore analyzed expression of CD122, the IL-2 receptor β-chain, on CD8<sup>+</sup> T cells (Fig. 1b). All CD8<sup>+</sup> T cells expressed CD122, albeit most at low levels. Whereas all CD8<sup>+</sup>CD28<sup>-</sup> cells were CD122<sup>low</sup>, a fraction of CD8<sup>+</sup>CD28<sup>+</sup> cells expressed high levels of CD122. Inversely, CD122<sup>high</sup> cells all expressed very high levels of CD28 (not shown). We also analyzed expression of markers that allow for distinction of naïve, activated, and memory T cells (Fig. 1b). Among CD28<sup>+</sup> cells a population of CD44<sup>high</sup> activated/memory CD8<sup>+</sup> T cells was found. CD44<sup>high</sup> cells expressed high levels of CD122 (not shown). In contrast, CD28<sup>-</sup> cells were all CD44<sup>low</sup>. No difference in CD45RB expression between CD28<sup>+</sup> vs. CD28<sup>-</sup> CD8<sup>+</sup> T cells was observed, and these cells were mostly CD45RB<sup>high</sup>. Moreover, CD8<sup>+</sup>CD28<sup>-</sup> T cells were mostly CD25<sup>low</sup> and CD62L<sup>high</sup>. Therefore, CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells had a naïve quiescent phenotype and were clearly distinct from regulatory CD8<sup>+</sup>CD122<sup>+</sup> T cells.

### *Freshly isolated CD8<sup>+</sup>CD28<sup>-</sup> cells inhibit proliferation and IFN- $\gamma$ production by CD4<sup>+</sup> T cells*

CD8<sup>+</sup>CD28<sup>-</sup> T lymphocytes were isolated from wildtype mice and tested for their capacity to inhibit proliferation and IFN- $\gamma$  production by CD4<sup>+</sup> responder cells in allogeneic mixed lymphocyte cultures (Fig. 2). Splenocytes were depleted of CD4<sup>+</sup>, Fc $\gamma$ RIII<sup>+</sup>, and MHC class II<sup>+</sup> cells, and remaining cells were sorted by flow-cytometry based on expression of CD8 and CD28 (Fig. 2a). Freshly isolated C57BL/6 (B6, H-2<sup>b</sup>) CD4<sup>+</sup> T cells were stimulated with DBA/2 (H-2<sup>d</sup>) antigen-presenting cells *in vitro* in presence of CD8<sup>+</sup>CD28<sup>+</sup> or CD8<sup>+</sup>CD28<sup>-</sup> T cells, and proliferation and IFN- $\gamma$  production was measured. As shown in figure 2b, CD8<sup>+</sup>CD28<sup>-</sup> (but not CD8<sup>+</sup>CD28<sup>+</sup>) cells inhibited proliferation in these cultures (as measured by <sup>3</sup>H-thymidine incorporation). CD8<sup>+</sup>CD28<sup>-</sup> cells acted in a dose dependent manner and close to maximum suppression of proliferation was already observed at a CD8<sup>+</sup>CD28<sup>-</sup> to CD4 cell ratio of 1 to 8 (Fig. 2c). Next, we evaluated the capacity of CD8<sup>+</sup>CD28<sup>-</sup> cells to inhibit production of IFN- $\gamma$  (which is crucial for induction of experimental IBD in immunodeficient mice<sup>12</sup>) by CD4<sup>+</sup> cells. Addition of CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells to allogeneic mixed lymphocyte cultures resulted in a reduction to background levels of the frequency of IFN- $\gamma$  producing cells among CD4<sup>+</sup> T cells (Fig. 2d). In contrast, CD8<sup>+</sup>CD28<sup>+</sup> T cells did not inhibit differentiation of IFN- $\gamma$  producing alloreactive CD4<sup>+</sup> effector T cells (Fig. 2d). These data show that freshly isolated CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells efficiently inhibit proliferation and IFN- $\gamma$  production by CD4<sup>+</sup> responder T cells.

### *Activated CD8<sup>+</sup>CD28<sup>-</sup> cells produce the immunosuppressive cytokines IL-10 and TGF- $\beta$*

Immunomodulation by several regulatory T cell populations involves IL-10 and TGF- $\beta$ . We therefore investigated if CD8<sup>+</sup>CD28<sup>-</sup> cells can produce these cytokines. Regulatory T cells

were isolated from spleen and activated in presence of MHC-deficient APC and anti-CD3 $\epsilon$  antibody *ex vivo*. After one week of culture, T cells were restimulated with PMA/ionomycin in presence of the Golgi-blocker Brefeldin-A, and subsequently stained intracellularly with an antibody specific for IL-10. We observed that a substantial proportion (15 and 20% in two independent experiments) of activated CD8<sup>+</sup>CD28<sup>-</sup> cells produced IL-10 (Fig. 3a). We also evaluated production of TGF- $\beta$  by *ex vivo* activated CD8<sup>+</sup>CD28<sup>-</sup> cells. Latency Associated Peptide (LAP) is a proteolytic product of the pro TGF- $\beta$ 1 protein and its surface expression is therefore limited to TGF- $\beta$ 1 expressing cells<sup>32</sup>. As shown in figure 3a, a substantial proportion (20 and 25% in two independent experiments) of activated CD8<sup>+</sup>CD28<sup>-</sup> cells expressed LAP. Combined, these data show that *ex vivo* activated CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells expressed IL-10 and TGF- $\beta$ 1.

#### *In vitro* suppression by CD8<sup>+</sup>CD28<sup>-</sup> T cells does not require IL-10 or TGF- $\beta$

We next assessed if IL-10 and/or TGF- $\beta$  are involved in suppression of T cell activation by CD8<sup>+</sup>CD28<sup>-</sup> T cells *in vitro*. Phenotypic analysis of splenocytes (using the same markers as those used in Fig. 1b) revealed no difference between CD8<sup>+</sup>CD28<sup>-</sup> cells from wt and IL-10 deficient mice (data not shown). CD4<sup>+</sup> T cells were stimulated *in vitro* with MHC-deficient APC plus anti-CD3 $\epsilon$  antibody, in absence or presence of wt or IL-10 deficient CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells (at a one-to-one ratio), and proliferation in these cultures was analyzed three days later by assessment of <sup>3</sup>H-Thymidine incorporation (Fig. 3b). Wildtype, but also IL-10 deficient regulatory T cells very substantially inhibited proliferation. T cells from mice transgenic for a dominant negative form of TGF- $\beta$ RII (dnT $\beta$ RII) do not respond to TGF- $\beta$ <sup>29</sup>. Proliferation of dnT $\beta$ RII-transgenic CD4 responder T cells was substantially inhibited by wt regulatory T cells. However, in absence of IL-10 or of TGF- $\beta$  signaling, suppression of T cell

proliferation was less efficient than in their presence, suggesting that these cytokines may play a minor role in suppression of T cell responses *in vitro*.

Since in this experimental setup we could not distinguish between proliferation of regulatory CD8<sup>+</sup>CD28<sup>-</sup> and responder CD4<sup>+</sup> T cells, we also performed experiments in which proliferation of responder T cells could be assessed separately. Responder T cells were stained with the cytoplasmic dye CFSE which dilutes with every cell division. As shown in Fig. 3c, responder T cells cultured in presence of MHC deficient APC retained their very high level of CFSE staining and therefore had not proliferated. Addition of an anti-CD3ε antibody to such cultures resulted in dilution of CFSE staining, sign of strong proliferation of responder T cells. Wt, but also IL-10 deficient CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells efficiently inhibited proliferation of responders. Moreover, regulatory T cells efficiently inhibited proliferation of dnTβRII transgenic responder T cells (Fig. 3c). In conclusion, IL-10 production by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells and TGF-β responsiveness of responder T cells are not required for *in vitro* inhibition.

### *Freshly isolated CD8<sup>+</sup>CD28<sup>-</sup> T cells prevent experimental inflammatory bowel disease*

We next assessed if CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells can prevent experimental IBD in mice. IBD can be induced in immunodeficient mice by *i.v.* injection of syngeneic CD4<sup>+</sup>CD45RB<sup>high</sup> T lymphocytes. Three weeks post-transfer characteristic signs of IBD start to appear: weight-loss, diarrhea, and prostrated posture of the mice. Histological analysis of the colon usually shows significant polymorphonuclear and mononuclear cell-infiltration and hyperplasia of mucosa, severe elongation of crypts, and disappearance of goblet cells. Development of disease can be inhibited by injection of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T lymphocytes<sup>9</sup>.

We investigated if CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells have the capacity to prevent IBD induced by injection of CD4<sup>+</sup>CD45RB<sup>high</sup> in RAG-2 deficient C57BL/6 mice. For these experiments CD4<sup>+</sup>CD45RB<sup>high</sup>, CD8<sup>+</sup>CD28<sup>+</sup>, and CD8<sup>+</sup>CD28<sup>-</sup> T cells were sorted from fresh C57BL/6 splenocytes. CD4<sup>+</sup>CD45RB<sup>high</sup> cells alone (4x10<sup>5</sup>) or in combination with CD8<sup>+</sup>CD28<sup>-</sup> (or control CD8<sup>+</sup>CD28<sup>+</sup>) T cells (2x10<sup>5</sup>) were *i.v.* injected into RAG-2 deficient C57BL/6 hosts. The weight of the animals was monitored over a six-week period, after which the mice were euthanized and their colons subjected to histological analysis. As shown in figure 4a, mice injected with only CD4<sup>+</sup>CD45RB<sup>high</sup> cells substantially lost weight during this period. In contrast, mice co-injected with CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells did not lose weight. CD8<sup>+</sup>CD28<sup>+</sup> cells inhibited weight-loss somewhat, but considerably less so than CD8<sup>+</sup>CD28<sup>-</sup> cells.

Histological analysis of colons showed severe hyperplasia of colon mucosa in RAG-2 deficient mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells alone (Fig. 4b). We also observed near total disappearance of goblet cells and strong mononuclear and polymorphonuclear cell-infiltration. Occasionally, cryptic abscesses were seen in the colons of these mice (data not shown). In contrast, most RAG-2 deficient mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup> and CD8<sup>+</sup>CD28<sup>-</sup> cells did not show signs of IBD. Mice co-injected with control CD8<sup>+</sup>CD28<sup>+</sup> cells had the same or even exaggerated colonic anomalies as mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup> cells alone.

We also graded pathology using histological colon-sections colored with hematoxylin and eosin (Fig. 4c). This analysis showed that CD8<sup>+</sup>CD28<sup>-</sup> cells efficiently protected mice from histological signs of IBD. In contrast, despite the only moderate weight loss in mice injected with CD45RB<sup>high</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells, grading of pathology revealed at least as severe colitis as in mice that had been injected with CD45RB<sup>high</sup> cells alone.

### *Prevention of IBD requires IL-10 production by CD8<sup>+</sup>CD28<sup>-</sup> cells*

Experimental IBD can also be prevented by injection of regulatory T cells of CD4<sup>+</sup>CD25<sup>+</sup> phenotype<sup>9</sup>. CD4<sup>+</sup>CD25<sup>+</sup> T cells from IL-10 deficient mice did not prevent colitis, demonstrating the non-redundant role of this anti-inflammatory cytokine in prevention of IBD<sup>13</sup>. We therefore evaluated the role of IL-10 in CD8<sup>+</sup>CD28<sup>-</sup> T cell-mediated prevention of colitis. RAG-2 deficient mice were injected with CD4<sup>+</sup>CD45RB<sup>high</sup> and wildtype or IL-10 deficient CD8<sup>+</sup>CD28<sup>-</sup> cells. Mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup> and IL-10 deficient CD8<sup>+</sup>CD28<sup>-</sup> cells lost as much weight as mice injected with colitogenic CD4<sup>+</sup>CD45RB<sup>high</sup> cells alone (Fig. 5a). Histological analysis of the colons of these mice 6 weeks after transfer showed no difference between mice injected with colitogenic cells alone or in combination with IL-10 deficient CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells (Fig. 5b). Clinical grading of colitis in these mice confirmed that IL-10 deficient CD8<sup>+</sup>CD28<sup>-</sup> cells did not protect against experimental IBD (Fig. 5c). We conclude therefore that IL-10 production by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells plays a crucial and non-redundant role in prevention of experimentally induced colitis.

### *TGF-β responsiveness of colitogenic T cells is required for CD8<sup>+</sup>CD28<sup>-</sup> T cell-mediated prevention of IBD*

Since TGF-β plays an important role in regulation of immune responses, including CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell-mediated prevention of colitis<sup>14,32</sup>, we also evaluated the involvement of this cytokine in the CD8<sup>+</sup>CD28<sup>-</sup> T cell-mediated prevention of IBD. When injected into RAG-2 deficient hosts, dnTβRII-transgenic CD4<sup>+</sup>CD45RB<sup>high</sup> cells induced weight loss and colitis (Fig. 6). Co-injection of wildtype CD8<sup>+</sup>CD28<sup>-</sup> T cells failed to reduce weight-loss (Fig. 6a). Histological analysis revealed clear signs of colitis in mice injected with dnTβRII CD4<sup>+</sup>CD45RB<sup>high</sup> colitogenic T cells. Co-injection of CD8<sup>+</sup>CD28<sup>-</sup> regulatory cells

with dnT $\beta$ RII transgenic colitogenic cells did not prevent these signs (Fig. 6b). Grading of colitis firmly established that CD8<sup>+</sup>CD28<sup>-</sup> T cells did not prevent colitis induced with dnT $\beta$ RII transgenic T cells (Fig. 6c). These data show that TGF- $\beta$  plays a crucial and non-redundant role in prevention of colitis by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T lymphocytes.

*CD8<sup>+</sup>CD28<sup>-</sup> T cells isolated from intestinal epithelium and lamina propria prevent experimental IBD*

The data presented here suggest that CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells may be involved in the physiological control of intestinal immunity. To more directly address this issue, we isolated CD8<sup>+</sup>CD28<sup>-</sup> (and CD8<sup>+</sup>CD28<sup>+</sup>) LPL and IEL from normal intestines and evaluated their capacity to prevent experimentally induced IBD (Fig. 7). Flow-cytometry analysis of CD8<sup>+</sup>TCR $\beta$ <sup>+</sup> IEL revealed a clearly distinguishable population of CD28<sup>-</sup> cells (Fig. 7a). A lower proportion of CD28<sup>-</sup> cells was found among CD8<sup>+</sup>TCR $\beta$ <sup>+</sup> LPL. CD28<sup>-</sup> and CD28<sup>+</sup> CD8<sup>+</sup> cells ( $2 \times 10^5$ ) isolated from LPL and IEL were co-injected with colitogenic CD4<sup>+</sup>CD45RB<sup>high</sup> cells ( $4 \times 10^5$ ) into RAG-2 deficient hosts. Mice injected with colitogenic cells alone lost weight over the six weeks following reconstitution. In contrast, mice co-injected with colitogenic cells and CD8<sup>+</sup>CD28<sup>-</sup> (but not CD8<sup>+</sup>CD28<sup>+</sup>) LPL or IEL increased their weight (Fig. 7b). At six weeks, mice were euthanized and their colons analyzed by histology (Fig. 7c). Colons of mice reconstituted with colitogenic CD4<sup>+</sup>CD45RB<sup>high</sup> cells alone showed clear signs of IBD, most dramatically severe mucosal hyperplasia. Colons from mice co-injected with colitogenic cells and CD8<sup>+</sup>CD28<sup>-</sup> (but not CD8<sup>+</sup>CD28<sup>+</sup>) LPL or IEL looked mostly healthy. Scoring of colitis in the five different experimental groups revealed a clear protection from IBD in mice co-injected with CD8<sup>+</sup>CD28<sup>-</sup> (but not CD8<sup>+</sup>CD28<sup>+</sup>) LPL or IEL (Fig. 7d). Combined, these data show that intestinal CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells prevented colitis and

strongly suggest that these cells may be involved in regulating intestinal immune responses in physiological conditions.

## Discussion

We here demonstrated that CD8<sup>+</sup>CD28<sup>-</sup> T lymphocytes from unmanipulated wildtype mice efficiently inhibited proliferation and IFN- $\gamma$  production by CD4<sup>+</sup> responder T cells in allogeneic mixed lymphocyte cultures. Naïve CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells, isolated from spleen or intestines, efficiently inhibited inflammatory bowel disease induced by transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> cells into immunodeficient mice. This *in vivo* immunosuppression required IL-10 production by regulatory T cells and responsiveness to TGF- $\beta$  of colitogenic effector cells.

Phenotypic analysis of CD8<sup>+</sup>CD28<sup>-</sup> regulatory T lymphocytes clearly distinguished them from the previously reported immunomodulatory “CD8<sup>+</sup>CD122<sup>+</sup>” T cell population. In contrast to two earlier reports<sup>20,21</sup>, we found that practically all CD8<sup>+</sup> T cells expressed low but significant levels of the IL-2 receptor  $\beta$  chain, CD122. Based on the percentages of the distinct CD122-expressing populations, we think that the “CD8<sup>+</sup>CD122<sup>+</sup>” population described in these reports corresponded to CD8<sup>+</sup>CD122<sup>high</sup> cells. CD8<sup>+</sup>CD28<sup>-</sup> cells expressed low levels of CD122 and are therefore clearly different from the CD8<sup>+</sup>CD122<sup>high</sup> population. It appears therefore that in the mouse at least two distinct naturally occurring (i.e. non-induced) CD8<sup>+</sup> regulatory T cell populations exist.

CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells inhibited proliferation and IFN- $\gamma$  production by CD4<sup>+</sup> T cells in allogeneic mixed lymphocyte cultures. These cells also prevented inflammatory bowel disease induced by injection of CD4<sup>+</sup>CD45RB<sup>high</sup> cells into immunodeficient RAG-2 deficient mice. CD8<sup>+</sup>CD28<sup>-</sup> T cells have previously been described to reduce severity of experimental autoimmune encephalomyelitis<sup>18</sup>, but this is the first demonstration that they can efficiently prevent IBD. In Man, LP CD8<sup>+</sup> T cells from healthy controls but not from patients affected with IBD have suppressive activity *in vitro*<sup>10</sup>. Stimulation of peripheral blood T cells with

intestinal epithelial cells leads to proliferation of CD8<sup>+</sup>CD28<sup>-</sup> T cells with *in vitro* suppressive activity<sup>28</sup>. In mice, CD8αα (but not CD8αβ) IEL inhibited development of IBD induced with CD4<sup>+</sup>CD45RB<sup>high</sup> cells in SCID animals<sup>33</sup>. Whereas, given the very high number of regulatory T cells required and the timing of their administration, the physiological relevance of the latter report remains unclear, combined the cited reports strongly suggest that CD8<sup>+</sup> regulatory T cells play an important role in physiological control of intestinal immune responses.

In our study, CD8<sup>+</sup>CD28<sup>-</sup> but not CD8<sup>+</sup>CD28<sup>+</sup> T cells (from spleen, LPL, or IEL) prevented IBD. In contrast, both CD28<sup>+</sup> and CD28<sup>-</sup> CD8<sup>+</sup> cells from human LPL had *in vitro* suppressive activity<sup>10</sup>. Moreover, mouse CD8αα IEL, present in both the CD28<sup>+</sup> and the CD28<sup>-</sup> populations (our unpublished data), inhibited IBD in SCID mice<sup>33</sup>. These discrepancies probably reflect differences between Man and Mouse and between experimental setups. However, they emphasize the need for more detailed definition of the distinct regulatory T cell populations in the gut.

In our study, regulatory CD8<sup>+</sup>CD28<sup>-</sup> T lymphocytes were defined as those expressing CD28 at levels not exceeding background. While no clear CD28<sup>-</sup> population was distinguishable in spleen and LPL, the CD28<sup>-</sup> and CD28<sup>+</sup> populations were clearly discernible in IEL. A combination of the partial overlap of the FACS-curves of CD28<sup>-</sup> and CD28<sup>+</sup> CD8<sup>+</sup> T cells (most readily visible in Figs. 1a and 7a), and the limited number of CD28<sup>-</sup> cells among splenocytes and LPL, avoid their clear visualization by flow-cytometry. In contrast, the functional data clearly indicate that CD28<sup>-</sup> and CD28<sup>+</sup> cells are different. However, future identification of additional markers for CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells will be required to better define this population.

*Ex vivo* activated CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells expressed IL-10. Whereas IL-10 played only a very minor (if any) role in *in vitro* suppression of T cell activation, IL-10 production by

CD8<sup>+</sup>CD28<sup>-</sup> cells played a crucial role in prevention of IBD. The discrepancy between requirement for IL-10 in *in vitro* vs. *in vivo* suppression by CD8<sup>+</sup>CD28<sup>-</sup> cells indicates that distinct mechanisms are employed. It appears therefore that these regulatory T cells use multiple mechanisms of suppression. A similar discrepancy between requirement for IL-10 has previously been observed in CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell-mediated suppression<sup>13,34</sup>. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell-derived IL-10 has been shown to prevent IBD through control of innate and adaptive immune responses<sup>35</sup> and similar mechanisms are therefore probably employed by regulatory CD8<sup>+</sup>CD28<sup>-</sup> cells. In contrast to mouse CD8<sup>+</sup>CD28<sup>-</sup> regulatory T lymphocytes, human “suppressor” CD8<sup>+</sup>CD28<sup>-</sup> T cells (obtained by repeated *in vitro* stimulation of peripheral blood lymphocytes with allogeneic APC) do not produce IL-10<sup>17</sup>. Several naturally occurring and induced CD8<sup>+</sup> regulatory T cell populations producing IL-10 have been identified<sup>17,21,27</sup>. Therefore, most but not all CD8<sup>+</sup> regulatory T cell-populations appear to produce IL-10. It will be of interest to assess if *in vivo* regulation of immune responses by the distinct populations requires IL-10. *In vivo* immunoregulation by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells is thought to depend on IL-10 only in case a substantial inflammatory component is involved in the experimental setting used<sup>36</sup>. It will therefore be important to carefully select experimental models used to evaluate the involvement of IL-10 in CD8<sup>+</sup> T cell-mediated regulation of *in vivo* immune responses.

Our data show that prevention of colitis by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T lymphocytes required TGF-β responsiveness of colitogenic effector cells. TGF-β blocks T cell proliferation as well as Th1 and Th2 differentiation<sup>32</sup>, which probably explains our observations. Another, not exclusive potential mechanism whereby TGF-β may prevent IBD, is the induction of Foxp3-expression in CD4<sup>+</sup>CD25<sup>-</sup> T cells by this cytokine<sup>37</sup>. Expression of this transcription factor induces regulatory function of T lymphocytes<sup>38-40</sup>. Thus, CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cell-derived

TGF- $\beta$  may induce other regulatory T cell populations that could contribute to control of intestinal immunity.

Whereas CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells expressed TGF- $\beta$ 1 (as assessed by analysis of cell-surface LAP) after *in vitro* stimulation, this does not necessarily mean that these cells express the TGF- $\beta$  involved in prevention of IBD. Similar to our results, it has previously been reported that mouse CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells produced TGF- $\beta$ 1<sup>41</sup> and that prevention of colitis by these cells required TGF- $\beta$  responsiveness of colitogenic T cells<sup>14</sup>. However, in the latter study, TGF- $\beta$ 1 production by CD4<sup>+</sup>CD25<sup>+</sup> cells was not required for prevention of IBD. It was therefore hypothesized that regulatory T cells may induce production of this cytokine by other cells<sup>14</sup>. In contrast, regulatory T cells from TGF- $\beta$ 1 deficient mice did not inhibit colitis in another report<sup>41</sup>. Moreover, LAP<sup>+</sup> but not LAP<sup>-</sup> CD4<sup>+</sup> T cells prevented colitis<sup>41</sup>. Given these contradictory reports on TGF- $\beta$  in CD4<sup>+</sup> regulatory T cell-based prevention of colitis, it will be important to evaluate the precise mechanisms involved in the TGF- $\beta$  dependent prevention of colitis by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells.

In contrast to the requirement for TGF- $\beta$  signaling in prevention of IBD by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells, these cells suppressed responder T cell proliferation in a TGF- $\beta$  independent manner *in vitro*. The discrepancy between requirement for TGF- $\beta$  in CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cell-mediated suppression *in vitro* and *in vivo* again indicates that these cells make use of multiple suppressor-effector mechanisms. The same *in vitro* vs. *in vivo* discrepancy has previously been described for CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells<sup>14,41-44</sup>. However, it has also been described that TGF- $\beta$  is required for *in vitro* suppression of T cell activation by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells<sup>45</sup>. Similar to our results, *in vitro* inhibition of T cell activation by mouse CD122<sup>high</sup> or human DC2-induced CD8<sup>+</sup> regulatory T cells did not require TGF- $\beta$ <sup>21,26</sup>. In contrast, less well defined CD8<sup>+</sup> regulatory T cells functioning in a TGF- $\beta$  dependent manner *in vitro* have previously been reported<sup>46-48</sup>. Therefore, distinct CD8<sup>+</sup>

regulatory T cell populations may function in different ways. However, involvement of TGF- $\beta$  in *in vivo* suppression of immune responses by the distinct CD8<sup>+</sup> regulatory T cell populations will need to be studied before meaningful conclusions can be drawn.

As stated above, our results showing that IL-10 and TGF- $\beta$  are required for prevention of colitis but do not play a crucial role *in vitro* indicate that CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells make use of multiple suppressor-effector mechanisms. Several suppressor mechanisms are also known to be used by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. These mechanisms include production of IL-10 and induction of TGF- $\beta$  production, but also expression of CTLA-4. CTLA-4 interacts with CD80 and CD86 expressed by antigen-presenting cells and by effector T cells thereby suppressing T cell activation<sup>49-51</sup>. Interestingly, interaction of CTLA-4 with CD80/CD86 expressed by effector T cells is the only mechanism known to be involved in *in vitro* inhibition of T cell activation by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells<sup>52</sup>. It will be important to further study which mechanisms are employed by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells and to gain insight into at what stage which suppressor functions intervene.

In conclusion, CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells inhibited IFN- $\gamma$  production *in vitro* and prevented experimentally induced inflammatory bowel disease. IL-10 and TGF- $\beta$  played crucial and non-redundant roles in the latter process. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells appear to use the same suppressor effector mechanisms in prevention of colitis<sup>13,14,41</sup>. It will therefore be important to study to what extent CD4<sup>+</sup>CD25<sup>+</sup> and CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells have similar characteristics. The ever-growing definition of distinct regulatory T cell populations that can be isolated from unmanipulated animals widens the avenue towards development of cell-based therapies against unwanted immune responses *in vivo*.

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## Figure Legends

**Figure 1** *Phenotypic analysis of CD8<sup>+</sup>CD28<sup>-</sup> splenocytes.* (a) C57BL/6 splenocytes were analyzed for expression of CD4, CD8 and CD28 by flow cytometry. CD28-expression by CD4<sup>+</sup> cells (thick gray line) and CD8<sup>+</sup> cells (thick black line) is shown. Thin lines represent background signal on CD8<sup>+</sup> (black) and CD4<sup>+</sup> (gray) splenocytes as determined with an isotype-matched control antibody. (b) Expression of indicated surface markers by electronically gated CD8<sup>+</sup>CD28<sup>-</sup> cells (thick gray line, gray shaded) and CD8<sup>+</sup>CD28<sup>+</sup> cells (thick black line). Thin lines indicate background staining on CD8<sup>+</sup>CD28<sup>-</sup> cells (gray) and CD8<sup>+</sup>CD28<sup>+</sup> cells (black).

**Figure 2** *Freshly isolated CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells inhibit proliferation and IFN- $\gamma$  production by CD4<sup>+</sup> responder T cells.* (a) CD28<sup>-</sup> and CD28<sup>+</sup> CD8<sup>+</sup> cells were sorted by flow cytometry as described in the Materials and Methods section. (b) Indicated cells were co-cultured and proliferation was determined by measuring incorporation of <sup>3</sup>H-Thy. (c) DBA/2 APC were cultured with 10<sup>5</sup> C57BL/6 CD4<sup>+</sup> and increasing numbers of CD8<sup>+</sup>CD28<sup>+</sup> splenocytes (at indicated ratios) and proliferation was determined by measuring incorporation of <sup>3</sup>H-Thy. Dashed line indicates background proliferation in presence of MHC-deficient APC. (d) Indicated cells were co-cultured and after three days cells were analyzed for expression of CD4 and production of IFN- $\gamma$  by flow cytometry. Indicated numbers represent percentages of IFN- $\gamma$  producing cells among CD4<sup>+</sup> cells. Shown results are representative of those obtained in three independent experiments. Indicated are mean values  $\pm$  SD (triplicates), \*\*\*P<0.001 (Student's t-test).

**Figure 3** *CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells express IL-10 and TGF-β1 but these cytokines are not required for their suppressive activity in vitro.* (a) Sorted CD8<sup>+</sup>CD28<sup>-</sup> splenocytes were activated *in vitro* with anti-CD3ε antibody during one week and then stained with antibodies specific for IL-10 or pro-TGF-β1 derived LAP (gray line with gray shading), or with isotype matched control antibodies (black line without shading). (b) Indicated responder CD4<sup>+</sup> T cells were cultured in presence of indicated CD8<sup>+</sup>CD28<sup>-</sup> suppressor cells and anti-CD3ε antibody. Proliferation in the cultures was determined by assessment of <sup>3</sup>H-Thy incorporation. Shown results are representative of those obtained in two independent experiments. Indicated are mean values ± SD (triplicates), \*\*\*P<0.001 (Student's t-test). T cell proliferation was less efficiently inhibited by IL-10 deficient than by wt CD8<sup>+</sup>CD28<sup>-</sup> cells (P<0.05, Student's t-test). DnTβRII responders were less efficiently inhibited than wt responders by CD8<sup>+</sup>CD28<sup>-</sup> cells (P<0.05, Student's t-test). (c) As in b, but responder cells were CFSE-labeled before culture and proliferation was assessed by FACS analysis of CFSE dilution on electronically gated CD4<sup>+</sup> responders. The dashed reference line is to indicate CFSE-signal on undivided cells. Shown are representative results from two independent experiments.

**Figure 4** *CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells prevent development of colitis.* RAG-2 deficient C57BL/6 mice were injected with indicated cells. (a) Evolution of weight of animals. Shown is the mean weight ± SD (n=4 from one representative experiment out of three) as a percentage of weight at start of experiment, \*P<0.05 (Mann-Whitney test). (b) Mice were euthanized six weeks after injection of T cells. Microscopic sections of distal colon were stained with hematoxylin and eosin and examined for signs of colitis. Shown results are representative of those obtained in three independent experiments. (c) Colons of mice were

examined as in b and clinical scores of colitis attributed as described in Materials and Methods section (n=12 from three independent experiments).

**Figure 5** *IL-10 production by CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells is required for prevention of colitis.* RAG-2 deficient C57BL/6 mice were injected with indicated cells. (a) Evolution of weight of animals. Shown is the mean weight  $\pm$  SD (n=4 from one representative experiment out of three) as a percentage of weight at start of experiment, \*P<0.05 (Mann-Whitney test). (b) Mice were euthanized six weeks after injection of T cells. Microscopic sections of distal colon were stained with hematoxylin and eosin and examined for signs of colitis. Shown results are representative of those obtained in three independent experiments. (c) Colons of mice were examined as in b and clinical scores of colitis attributed as described in Materials and Methods section (n=12 from three independent experiments).

**Figure 6** *CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells do not prevent colitis induced with CD4<sup>+</sup>CD45RB<sup>high</sup> cells incapable of responding to TGF- $\beta$ .* RAG-2 deficient C57BL/6 mice were injected with indicated cells. (a) Evolution of weight of animals. Shown is the mean value  $\pm$  SD (n=5) as a percentage of weight at start of experiment, \*P<0.05 (Mann-Whitney test). Data from a representative experiment out of two are depicted. (b) Mice were euthanized six weeks after injection of T cells. Microscopic sections of distal colon were stained with hematoxylin and eosin and examined for signs of colitis. Shown results are representative of those obtained in two independent experiments. (c) Colons of mice were examined as in b and clinical scores of colitis attributed as described in Materials and Methods section (n=11 from two independent experiments).

**Figure 7** *CD8<sup>+</sup>CD28<sup>-</sup> (but not CD8<sup>+</sup>CD28<sup>+</sup>) IEL and LPL efficiently prevent development of colitis.* (a) CD28-expression by (gray shading), or background staining on (black line) electronically gated CD8<sup>+</sup>TCRβ<sup>+</sup> cells. RAG-2 deficient C57BL/6 mice were injected with indicated populations. (b) Evolution of weight of animals. Shown is the mean weight ± SD (n=7 from 3 independent experiments) as a percentage of weight at start of experiment, \*P<0.05 (Mann-Whitney test). (c) Mice were euthanized six weeks after injection of T cells. Microscopic sections of distal colon were stained with hematoxylin and eosin and examined for signs of colitis. Shown results are representative of those obtained in three independent experiments. (d) Colons of mice were examined as in c and clinical scores of colitis attributed as described in Materials and Methods section (n=7, from 3 independent experiments).

Figure 1

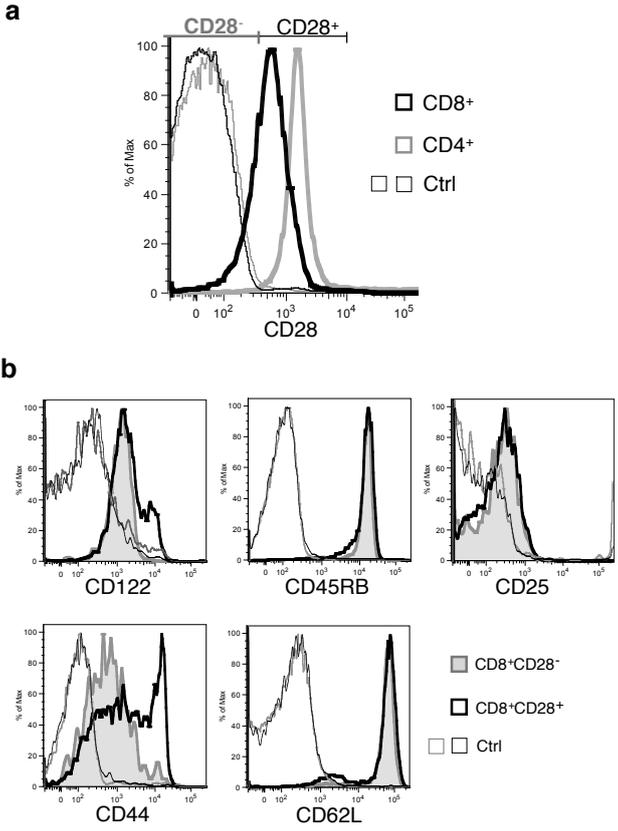


Figure 2

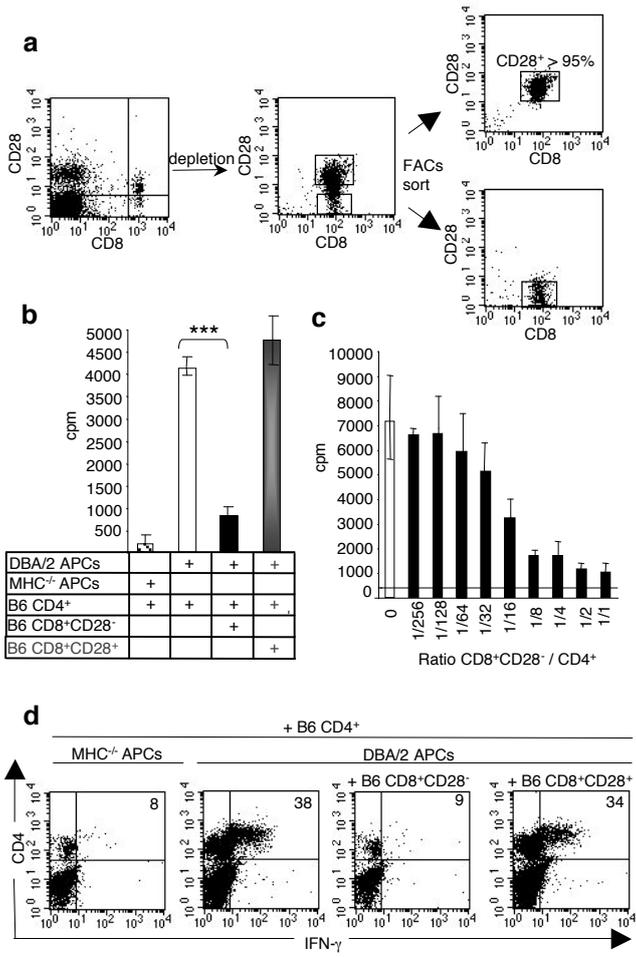


Figure 3

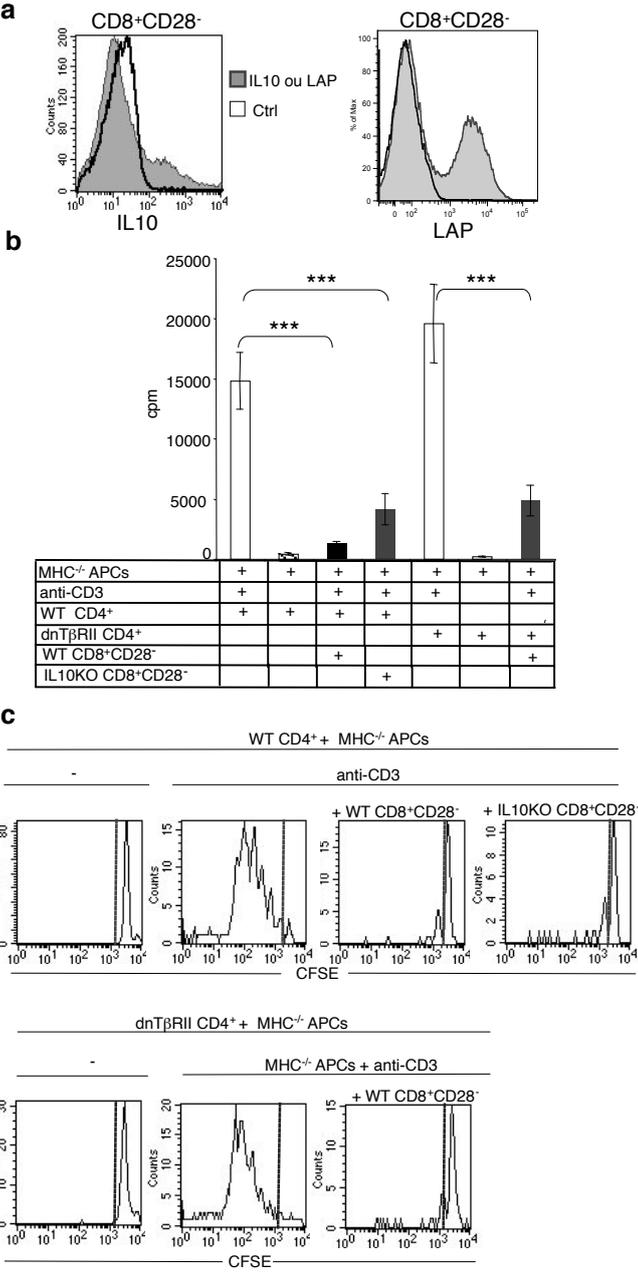


Figure 4

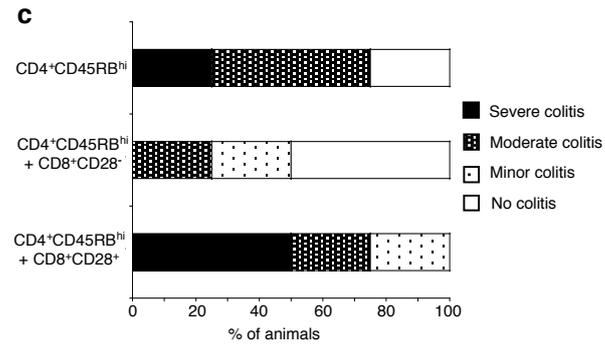
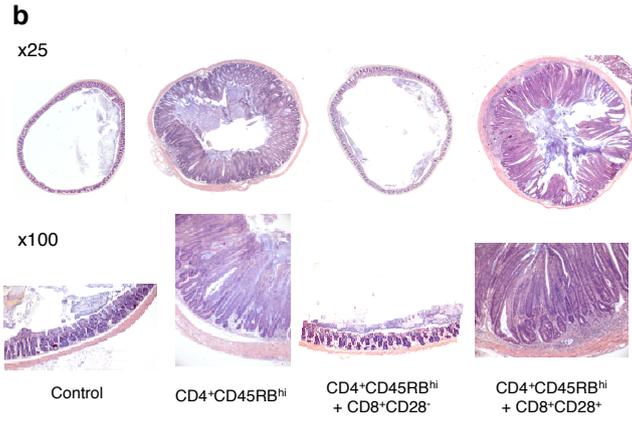
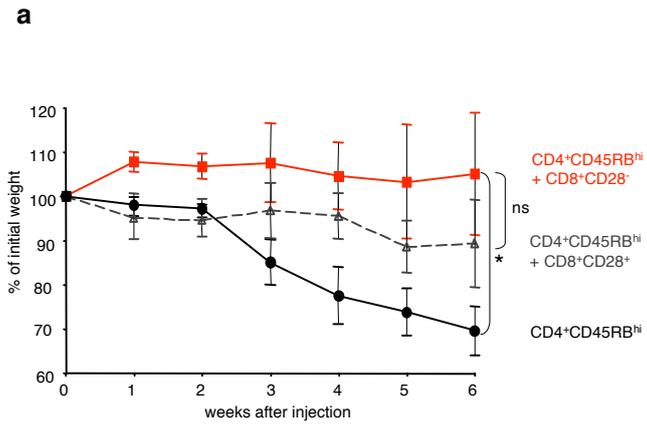


Figure 5

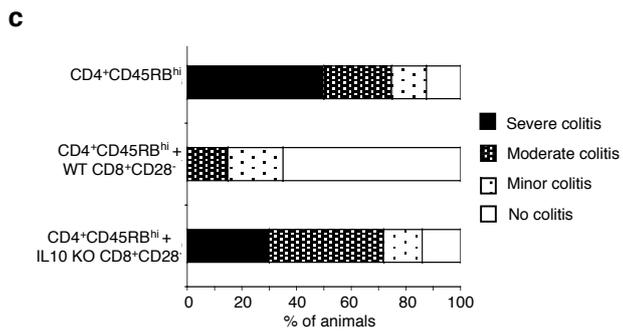
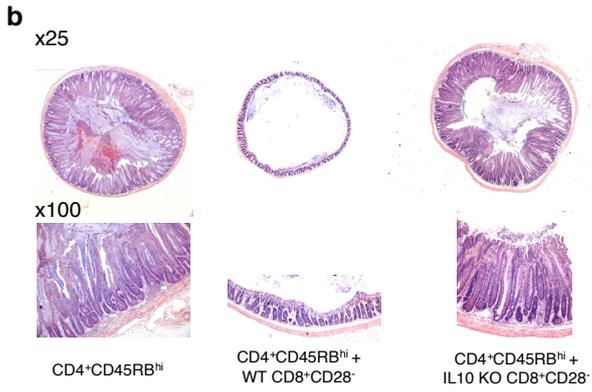
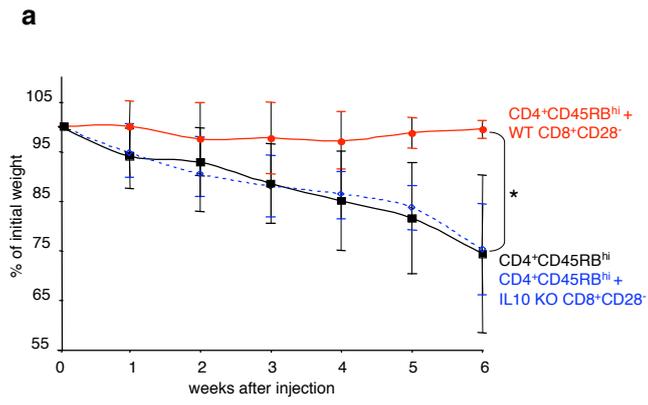


Figure 6

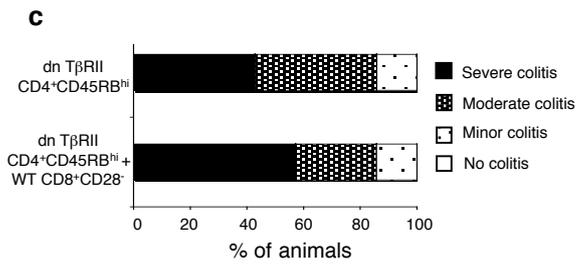
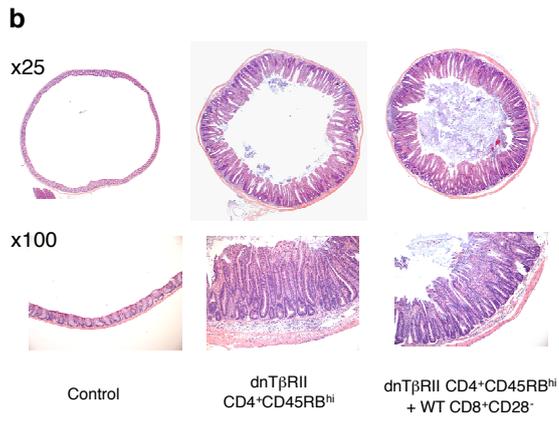
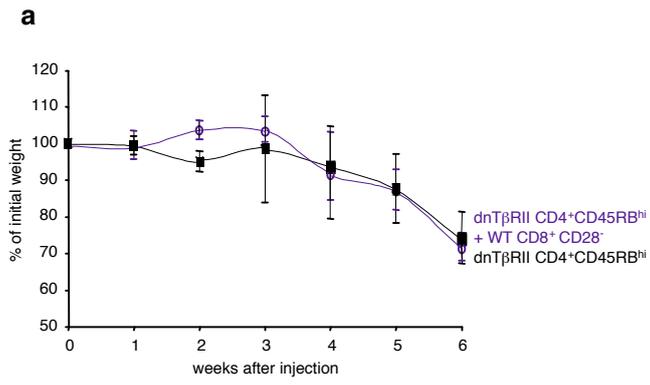


Figure 7

