An important regulatory role for CD4$^+$CD8$\alpha\alpha$ T cells in the intestinal epithelial layer in the prevention of inflammatory bowel disease

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The normal immunoregulatory mechanisms that maintain homeostasis in the intestinal mucosa, despite continuous provocation by environmental antigens, are jeopardized in inflammatory bowel diseases. Although previous studies have suggested that intestinal intraepithelial lymphocytes prevent spontaneous intestinal inflammation, there is limited knowledge about the characteristics of regulatory cells in the intestinal intraepithelial lymphocytes population. Here we show that CD4$^+$CD8$\alpha\alpha^+$ double-positive cells present in the intestinal intraepithelial lymphocytes population can suppress T helper 1-induced intestinal inflammation in an IL-10-dependent fashion. CD4$^+$ T cells stimulated along the Th2 but not the Th1 lineage, when transferred to *RAG-1*−/− mice, acquire CD8$\alpha\alpha$ expression on reaching the intestinal epithelium, and on arrival there, augment their production of IL-10. We show that a precursor CD4$^+$ T cell after limited, but not repeated, stimulation by IL-4 is able to become a double-positive-regulatory cell on exposure to the intestinal microenvironment in mice. Both CD8$\alpha\alpha$ acquisition and IL-10 production depend critically on the NF-κB-GATA-3-axis that we have previously shown is essential for differentiation to the Th2 phenotype and for the induction of airway inflammation. Our studies identify a mechanism for the generation of regulatory T cells in the intestine that may play an important role in controlling inflammatory bowel disease.

Inflammatory bowel disease (IBD) is a chronic relapsing inflammation, involving the small intestine, the large intestine, or both, and whose etiology and pathogenesis are poorly understood. It is well established that IBD is a T cell-mediated autoimmune disease. Several studies have shown that neutralization of IL-12 by administration of anti-IL-12 (1) or neutralization of IFN-γ by injection of anti-IFN-γ (2) prevents the initiation of disease and/or blocks ongoing disease, illustrating the importance of Th1-mediated effects in the disease process. Similarly, *scid* mice reconstituted with CD45RB^{high} T cells have been shown to develop spontaneous colitis (2). However, CD45RB^{high} cells from Stat-4-deficient mice were unable to do so, indicating the role of IL-12-dependent T helper (Th) 1 cells in disease initiation (3). Interestingly, cotransfer of CD45RB^{low} cells with CD45RB^{high} cells to *scid* mice prevented initiation and progression of colitis, suggesting a suppressive effect of CD45RB^{low} cells on CD45RB^{high} cells during the pathogenesis of IBD (2). However, it is unlikely that these CD45RB^{low} cells are prototypical Th2 cells, because Th2 cells can also induce colitis, as shown in human ulcerative colitis and in the trinitro benzene sulfonic acid-induced colitis model (4–7). Furthermore, IL-4 has been shown to cause aggravation of intestinal inflammation (8). Taken together, these studies suggest that both subsets of helper CD4 cells, Th1 and Th2, may participate in inducing inflammation within the intestines.

TCR$\alpha\beta$-deficient mice develop spontaneous colitis (1), as do MHC class II-deficient mice (1), suggesting an important suppressive role for TCR$\alpha\beta^+$ CD4$^+$ T cells in the prevention of spontaneous colitis (1). Here we show that CD4$^+$CD8$\alpha\alpha^+$CD8β^-TCR$\alpha\beta^+$ T cells, a unique cell type that resides in the intestinal epithelial layer, possesses a regulatory function in the inhibition of Th1-induced intestinal inflammation. These cells produce IL-10, TGF-β, and IFN-γ, which resembles a cytokine pattern previously described for Treg cells that were generated by stimulation of murine CD4$^+$ T cells with IL-10 in culture (9). Our data suggest that a CD4$^+$ T cell either can be stimulated to become a CD4$^+$CD8$\alpha\alpha^+$ DP regulatory cell in the intestinal microenvironment or is irreversibly committed to a Th2 phenotype that is unable to acquire a regulatory phenotype. Both of these processes initially require GATA-3, NF-κB, and IL-4. However, at a later stage of development, clearly additional stimuli are needed to induce the development of double-positive (DP) cells in the intestinal environment.

Materials and Methods

Mice and Antibodies. C57BL/6 (B6), Rag−/− mice on a B6 background, IL-10−/− mice on a B6 background, NF-κB p50−/− on a mixed B6/129 background, and control B6/129 mice were purchased from The Jackson Laboratory. AND Vα11/Vβ3 $\alpha\beta$ TCR transgenic (Tg) mice were previously described (10) and were bred onto a B6 background. GATA-3 dominant negative (GATA-3 DN) were described earlier (11) and maintained in a specific pathogen-free colony at the Yale Animal Facility.

Rag2−/− mice on BALB/c background were purchased from Taconic Farms. In all experiments, Clicks' medium (Irvine Scientific, Santa Ana, CA) with 10% FCS was used. Purified anti-CD3 (145–2C11), anti-CD28 (R2/60.1.12), biotin-labeled anti-IFN-γ (XMG1.2), and anti-IFN-γ (HB-170) antibodies were generated in our core antibody facility. Anti-Cβ-quantum red/PE/FITC (H57–597) and anti-CD4-quantum red/FITC/PE (H129.19) were purchased from BD PharMingen, and anti-CD8α-PE/FITC (53–6.7) was purchased from Life Technologies (Rockville, MD).

Isolation of Intestinal Epithelial Lymphocytes and CD4 T Cells and Generation of Th1 and Th2 Cells. Intestinal epithelial lymphocytes were prepared as described earlier with a minor modification (12). Briefly, small intestines were harvested and washed by swirling in PBS. Mesentery and Peyer's patches were carefully removed. The intestines were cut longitudinally and then into 0.5-cm pieces. Intestinal pieces were agitated in 25 ml of extraction buffer (3% FCS/1 mM DTT/1 mM EDTA in PBS) for 30 min at 37°C. The slurry was passed through a loosely packed nylon wool column to remove the aggregates. The follow-through was layered on a discontinuous Percoll gradient (Amersham Pharmacia Biotech). This gradient was then centrifuged at 900 × *g* for 20 min. Cells at the interface of the 40/70% layer were collected and washed in staining buffer. Splenic CD4$^+$ T cells were isolated by positive selection using monoclonal antibodies to CD4 coupled with magnetic beads (MACS Miltenyi Biotec, Auburn, CA) in a MACS

Abbreviations: iIEL, intestinal intraepithelial lymphocytes; Tg, transgenic; DP, double-positive; TCR, T cell receptor; Ts, T suppressor; Treg, T regulatory; H&E, hematoxylin/eosin; Th*n*, T helper *n*.

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preparation column. $CD4^+$ T cells were stimulated under Th1 or Th2 differentiating conditions *in vitro* with plate-bound anti-CD3ε (2 μg/ml), anti-CD28 (2 μg/ml), and IL-2 (10 units/ml) along with either IL-12 (5 ng/ml) and anti-IL-4 (10 ng/ml), or with IL-4 (200 units/ml) and anti-IFN-γ, respectively. Enrichment of IL-4-producing cells was done by using an enrichment kit (MACS Miltenyi Biotec). For repeated stimulation, cells were recovered from the primary culture under Th2 conditions and washed in complete medium. Cells were restimulated with plate-bound anti-CD3 plus anti-CD28 antibody under Th2 culture conditions. Cells were stimulated at least three times.

Adoptive Transfers. Cells grown *in vitro* under either Th1 or Th2 conditions for 5 days were washed three times in PBS. They were then adoptively transferred i.v. After 30 days of transfer, cells were recovered from the intestinal epithelium for further studies.

Fluorescence-Activated Cell Sorter Staining and Analysis and Cell Sorting. Cells were suspended in staining buffer (3% FCS, 0.01% sodium azide in PBS) at a concentration of 10^7 cells per ml. Fifty microliters of cell suspension was incubated with directly conjugated antibodies for 30 min on ice. Cells were washed twice in staining buffer and fixed with 1% paraformaldehyde. Fluorescence intensities were measured by using FACScan (Becton Dickinson). For cell sorting experiments, cells recovered from the intestinal epithelium were stained with anti-cβ (H57), anti-CD8α, and anti-CD4 antibodies in complete medium on ice. Sorting was carried out by using FACStar Flowcytometer (Becton Dickinson).

Cytokine Production by $CD4^+CD8\alpha\alpha^+$ DP Cells. $CD4^+CD8\alpha\alpha^+$ DP T cells 0.5×10^6 sorted cells were used for preparation of mRNA. mRNA was isolated by using Trizol, and cDNA was synthesized by using Superscript and oligo dT according to the manufacturer's instuctions (Life Technologies). The cDNA was used for performing RT-PCR for individual cytokines.

IFN-γ and IL-4 were measured by ELISA. The antibodies that were used to capture IL-4 and IFN-γ were 11B11 and HB-170, respectively. Biotinylated antibodies were purchased from PharMingen Corporation. IL-10 was measured by using an ELISA kit (Endogen, Cambridge, MA). The lower limits of detection for the cytokines IL-4, IFN-γ, and IL-10 were 12.5 ng/ml, 6.25 ng/ml, and 111 pg/ml, respectively.

Histology. Small and large intestinal pieces were washed thoroughly in PBS/FCS and then fixed in periodate-lysine-paraformaldehyde. Tissues were embedded in paraffin and 5- to 6-μm sections were cut by using a cryostat. The sections were stained with hematoxylin/eosin (H&E) and examined microscopically. The sections were viewed in a blinded fashion, and the severity of disease was scored on the basis of cellular infiltration in the lamina propria and the submucosa and on the extent of crypt distortion/degeneration.

Results

$CD4^+CD8\alpha\alpha^+$ DP T Cells Are Found in the Intestinal Intraepithelial Lymphocytes (iIEL) Compartment and They Are Thymus Derived. TCR$\alpha\beta$-deficient and MHC class II-deficient mice spontaneously develop colitis (1), suggesting an important suppressive role for $TCR\alpha\beta^+CD4^+$ T cells in the prevention of spontaneous colitis (1). To characterize the resident $CD4^+$ T cells in the iIEL population, T cells were isolated from this population. Low numbers of $CD4^+$ T cells are generally found in the iIEL compartment. Gating on CD4 showed that 20–30% of these $CD4^+$ T cells also expressed CD8α but not CD8β (Fig. 1*A*). Typically, $CD4^+CD8\alpha\alpha^+$ DP $TCR\alpha\beta^+$ cells are not found in peripheral lymphoid organs. However, these cells have been detected under conditions of heightened immune response, as observed in patients with myasthenia gravis, rheumatoid arthritis, and leprosy (13–15). Furthermore, $CD4^+CD8\alpha\alpha^+$ DP cells from lepromatous leprosy have been shown to inhibit the antigen-specific proliferation of T cells (15).

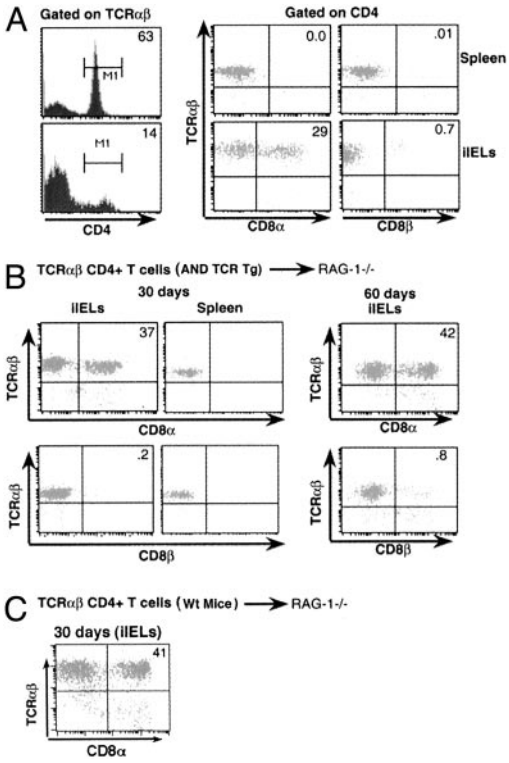

Fig. 1. CD4 T cells in the iIEL compartment acquire CD8α. (*A*) iIELs were harvested from C57BL/6 mice and stained for TCR and coreceptors. Gating on TCR$\alpha\beta$ showed that only a small number of CD4 T cells is found in the iIEL population in comparison to that in the spleen. Gating on CD4 showed that a large number of CD4 cells also express CD8α in iIELs but not in the spleen. However, none of these $CD4^+CD8\alpha^+$ DP cells express CD8β. (*B*) $TCR\alpha\beta\,CD4^+$ T cells were sorted from AND TCR Tg mice, activated with a combination of anti-CD3 and anti-CD28, and adoptively transferred to syngeneic *RAG-1*$-/-$ mice. CD4 T cells were harvested from the iIEL compartment on the indicated days and examined for coreceptor expression. A large number of CD4 cells acquired CD8α but not CD8β. (*C*) Splenic $TCR\alpha\beta\,CD4^+$ T cells were isolated from non-Tg mice, adoptively transferred to *RAG-1*$-/-$ mice and analyzed as in *B*.

The intestinal epithelial layer is the largest epithelial surface that continuously interacts with microbial flora and IBD requires microbial presence, because germ-free mice fail to develop symptoms. The presence of $CD4^+CD8\alpha\alpha^+$ DP cells in the intestinal layer suggested a role for these cells in suppressing inflammatory responses in the intestines, and we therefore decided to further investigate their activities.

Previous studies have shown that $CD4^+$ T cells found in the intestinal epithelial layer are thymus-derived (16). Because thymus-derived $CD4^+$ T cells do not express CD8 molecules in the periphery under normal conditions, it seemed likely that $CD4^+$ T cells, on reaching the intestinal epithelial layer, reacquire expression of CD8α as has been previously described in adoptive transfer experiments (17, 18). To test this hypothesis, anti-CD3 and anti-CD28-activated AND TCR Tg splenic $CD4^+$ T cells were transferred to syngeneic *RAG-1*-deficient mice. Four weeks after transfer, the Tg T cells were recovered from the intestinal epithelial layer and stained for the surface expression of both coreceptors. Thirty percent to 50% of the $CD4^+$ T cells recovered showed expression of CD8α but not CD8β (Fig. 1*B*). However, this was not observed in cells recovered from the spleen (Fig. 1*B*). This phenomenon was not limited to the AND TCR Tg $CD4^+$ T cells; sorted splenic $TCR\alpha\beta^+CD4^+$ T cells from C57BL/6 mice transferred to syngeneic *RAG-1*$-/-$ mice also showed similar CD8α expression when recovered from the intestinal epithelial layer (Fig. 1*C*). Of note, splenic $CD8^+$ T cells transferred to syngeneic *RAG-1*$-/-$ mice did

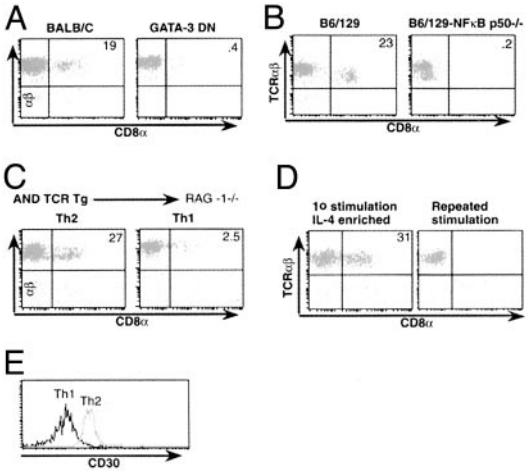

Fig. 2. Transcription factors NF-κB and GATA-3 are involved in the acquisition of CD8α on CD4 T cells in the iIEL compartment. (*A*) iIELs were isolated from GATA-3DN mice and stained for their coreceptors. Gating on CD4 shows that very few CD4 T cells express CD8α$^+$. (*B*) iIELs isolated from NF-κB p50−/− contain no CD4$^+$CD8αα$^+$ DP cells. (*C*) AND TCR Tg cells were cultured under Th1 and Th2 polarizing conditions for 3 days. They were then adoptively transferred to syngeneic *RAG-1*−/− mice. Four weeks posttransfer, they were harvested from iIEL compartment and stained for the expression of CD8α. Th2-polarized cells were able to acquire CD8α on their surface, whereas Th1-polarized cells failed to do so. (*D*) IL-4-producing cells either were enriched by positive selection after 4 days in culture under Th2-differentiating conditions (>80% enrichment) or were repeatedly stimulated under Th2 conditions and transferred to syngeneic RAG-1−/− mice. Thirty days after transfer, CD4 T cells were recovered from the iIEL compartment and analyzed for the expression of CD8α. IL-4-producing T cells from the primary culture yielded a considerable number of DP cells, whereas repeatedly stimulated cells could not generate DP cells in the iIELs. (*E*) CD4 T cells were cultured under either Th1 or Th2 polarizing conditions for 4 days. Cells were washed and restimulated under neutral conditions, and 48 h later they were stained with anti-CD30 antibody. Th2 cells showed a higher expression of CD30 and a single peak, whereas addition of anti-IL-4 and IFN-γ yielded essentially CD30-negative cells.

not down-regulate their CD8β expression nor did they express CD4 (data not shown). Thus, the DP cells are derived from a CD4$^+$ T cell lineage.

NF-κB and GATA-3 Are Required for the Development of CD4$^+$CD8αα$^+$ DP Treg Cells. CD4$^+$CD8$^+$TCRαβ$^+$ DP T cells are generally found in the thymus in the form of immature thymocytes. On maturation, these cells lose either CD4 or CD8, and this process is highly specific and tightly dependent on intrathymic positive selection of CD4 thymocytes on MHC class II or CD8 thymocytes on MHC class I molecules expressed on thymic epithelial cells. After positive and negative selection and the loss of CD8 molecules, CD4$^+$ T cells emigrate to the periphery and do not reexpress CD8 molecules in peripheral lymphoid tissues. The regulation of expression of these coreceptors in the thymus is a complex phenomenon and involves several factors. Previously, three GATA-3-binding sites were identified upstream of the CD8α gene, suggesting a role for this factor in CD8 gene expression (19). Also, in previous studies, we have shown that GATA-3 is critical in the development of a Th2 phenotype (11, 20). These observations, in combination with our data showing that peripheral CD4$^+$ T cells migrate to the intestinal epithelium and acquire CD8α on their surface (Fig. 1), led us to examine CD4$^+$CD8αα$^+$ DP T cells in Tg mice expressing dominant–negative (DN) GATA-3 (11). As shown in Fig. 2*A*, mice expressing DN GATA-3 were found to contain only few of these DP cells in their intestinal epithelial layer. Recently, we also showed that expression of GATA-3 is critically dependent on NF-κB; mice deficient in the p50 subunit of NF-κB neither express GATA-3 in their CD4$^+$ T cells, nor are they able to mount Th2 responses (21). As shown in Fig. 2B, analysis of p50−/− mice showed a complete absence of the CD4$^+$CD8αα$^+$ DP cells, whereas they were found in their wild-type littermates. Therefore, NF-κB and GATA-3 are involved, directly or indirectly, in the reexpression of CD8α molecules on the surface of mature CD4$^+$ T cells residing in the intestinal epithelial layer. It should also be noted that NF-κB p50−/− and GATA-3 DN mice contain normal numbers of CD8$^+$ T cells in the thymus and in the periphery (11, 22).

In the previous experiments, we established that GATA-3 and NF-κB are important for the acquisition of CD8α molecules on mature CD4$^+$ T cells residing in the intestinal epithelium. Because expression of GATA-3 and NF-κB contribute to the development of a Th2 axis, we examined the ability of Th1-polarized and Th2-polarized CD4$^+$ T cells to acquire CD8α while residing in this layer. To this end, Th1 and Th2 cells were generated from AND TCR Tg mice and adoptively transferred to syngeneic RAG-1−/− mice. Four weeks after transfer, Tg T cells were recovered from the iIEL compartment and analyzed for coreceptor expression. It was found that 20–30% of Th2 cells acquired CD8α on their surface, whereas Th1-polarized cells did not acquire CD8α expression (Fig. 2*C*). This observation further supports the involvement of GATA-3 and NF-κB in the acquisition of CD8α on the surface of mature CD4$^+$ T cells in the intestinal epithelial layer.

Because it was previously shown that not only Th1 but also Th2 cells can induce IBD, and DP cells are unable to produce IL-4 (23), we investigated the ability of CD4$^+$ T cells, maintained under Th2 culture conditions for different lengths of time, to generate DP cells in the iIEL compartment. Toward this end, we enriched Th2 cells by positive selection of IL-4-secreting cells from CD4$^+$ T cells maintained *in vitro* for 4 days under Th2-differentiating conditions and adoptively transferred them to syngeneic *RAG*−/− mice. Thirty days posttransfer, we were able to recover CD4$^+$CD8αα$^+$ DP cells from the iIEL compartment of the recipient mice (Fig. 2*D*). We further confirmed the Th2 direction of the cell population by assessing the status of CD30 expression on these cells because it was previously shown that IL-4-stimulated commitment to the Th2 lineage induced CD30 expression (24). As shown in Fig. 2*E*, a single peak of CD30-expressing cells was observed 48 h after secondary stimulation of the cells induced to develop along a Th2 pathway. This observation confirmed that the majority of the cells under our culture conditions were indeed stimulated toward the Th2 lineage, suggesting that the DP cells most likely pass through an IL-4-secreting phase before assuming an IL-4-negative CD4$^+$CD8αα$^+$ DP phenotype. This observation further suggested the possibility that the IL-4-containing cells after short-term culture have the plasticity to become either fully committed Th2 cells or under appropriate conditions, Treg cells. To test this hypothesis, we generated Th2 cells by repeated *in vitro* stimulation and adoptively transferred them to syngeneic *RAG*−/− mice. After 30 days, the CD4$^+$ T cells recovered from the iIEL compartment of the recipient mice did not express CD8αα (Fig. 2*D*). It was also found that these mice developed mild inflammation after cell transfer. Very recently, it has been shown that antigen-specific Th2 cells also induce intestinal inflammation (25). Taken together, these observations suggest that naïve CD4$^+$ T cells follow a common pathway of differentiation before becoming fully committed toward a Th2 or Treg endpoint. It will be interesting to determine whether Treg differentiation depends not only on the influence of the intestinal microenvironment but also on the level of GATA-3 protein expressed because the biological effects of GATA factors are thought to depend on their level of expression (26).

CD4$^+$CD8αα$^+$ DP T Cells Show a Treg Cytokine Pattern. CD4$^+$-CD8αα$^+$ DP T cells have been identified in the intestinal epithelial layer. Although the functions of these cells are unknown, freshly isolated CD4$^+$ CD8αα$^+$ T cells have been shown to express some Th2-type cytokines such as IL-5 and -6, but they do not produce IL-4, an important Th2 cytokine (23). This information, in com-

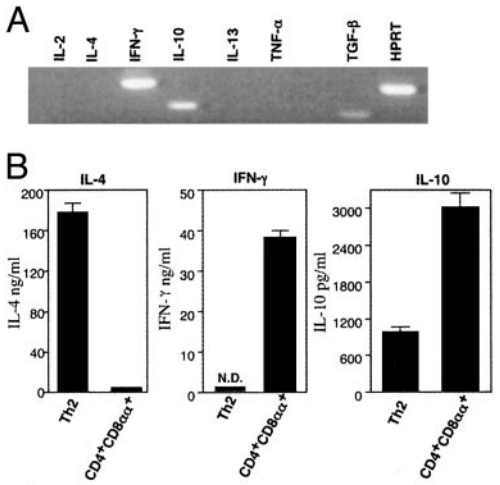

Fig. 3. $CD4^+CD8\alpha\alpha^+$ DP iIELs possess a cytokine profile similar to regulatory (Treg) or suppressor T cells (Ts). (*A*) $CD4^+CD8\alpha\alpha^+$ DP cells were sorted by using a FACSVantage sorter. RNA was isolated from 0.5×10^6 cells and used for RT-PCR for indicated cytokines by using specific primer pairs. (*B*) AND TCR Tg Th2-polarized cells were generated by culturing under Th2 conditions and their cytokine profile was measured by ELISA. They were transferred to *RAG-1*−/− mice. Four weeks after transfer, Tg cells were harvested from the iIEL compartment and $CD4^+CD8\alpha\alpha^+$ DP cells were sorted by using FACSVantage. They were activated with anti-CD3 + anti-CD28 along with IL-2. Forty-eight hours later, supernatants were harvested tested for their cytokine profile by ELISA.

bination with our data, suggested that Th2 cells, on reaching the intestinal environment, alter not only their cell surface expression but also their cytokine profile. We examined the cytokine expression of iIEL DP cells by RT-PCR. As shown in Fig. 3*A*, these cells expressed IL-10, TGF-β, and IFN-γ mRNA and resembled the previously described T regulatory cells (Tr1) or T suppressor (Ts) cells (9). Tr1 cells were originally generated by culturing $CD4^+$ T cells in the presence of IL-10 (9). To extend this observation to our adoptive Th2 transfer model, we determined the expression of IL-4, IL-10, and IFN-γ produced by the $CD4^+CD8\alpha\alpha^+$ DP cells extracted from the intestinal epithelium of *RAG-1*−/− mice, which were reconstituted with AND TCR Th2-polarized cells 4 weeks earlier. On activation, the DP cells produced increased amounts of IL-10 as compared with the original Th2 cells (Fig. 3*B*). They also produced a moderate amount of IFN-γ, which was undetectable in the original Th2 cells. Finally, these cells did not produce any IL-4 (Fig. 3*B*).

$CD4^+CD8\alpha\alpha^+$ DP Treg Cells Inhibit Th1-Induced Intestinal Inflammation in an IL-10-Dependent Fashion. Having demonstrated a Tr1 phenotype of the DP cells derived from the iIEL population, and given the potent suppressive role of Tr1 cells in $CD4^+CD45RB^{hi}$-induced colitis in *scid* mice (9), we examined the ability of these DP cells to inhibit Th1-induced intestinal inflammation. DP cells were sorted from *RAG-1*−/− mice that were previously reconstituted with Th2-polarized cells. Syngeneic *RAG-1*−/− mice were reconstituted with 1×10^6 sorted DP cells and challenged with 3×10^6 Th1 cells, a number of Th1 cells sufficient to induce gut inflammation. After 4 weeks, mice were killed, and both small and large intestines were fixed, embedded, and sections were cut, and these sections were examined by staining with H&E. Mice that were reconstituted with Th1 cells showed severe epithelial hyperplasia with severe inflammation in the submucosa (Fig. 4 *B* and *F*). On the contrary, mice that were reconstituted with polarized Th2 cells did not show these same pathological signs of disease (Fig. 4 *A* and *E*). Similarly, mice that were previously reconstituted with $CD4^+CD8\alpha\alpha^+$ DP cells showed no disease, resembling their wild-type littermates (Fig. 4 *C* and *G*).

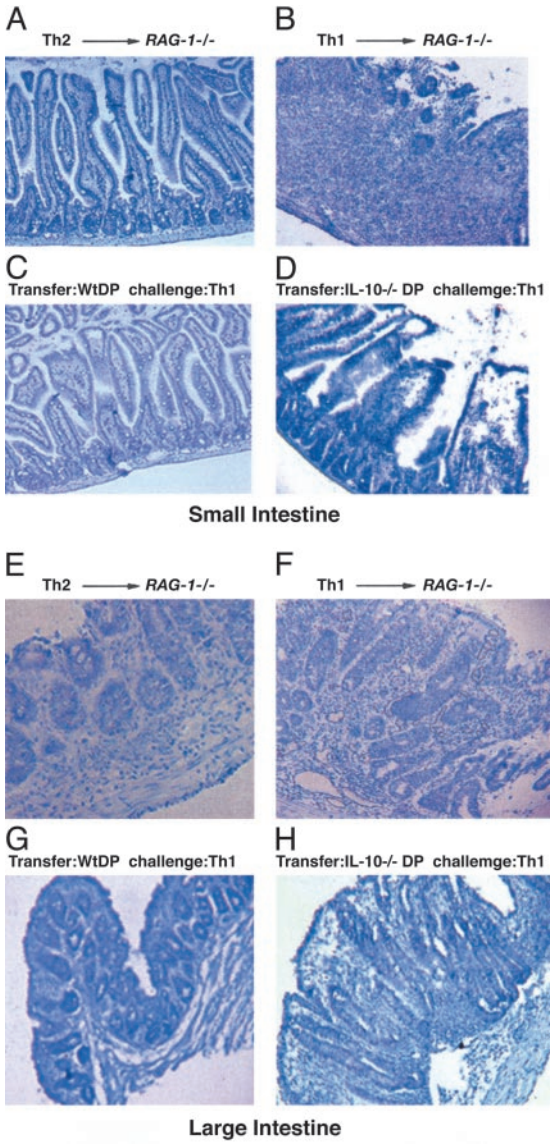

Fig. 4. Reciprocal relationship of Th1 and $CD4^+CD8\alpha\alpha^+$ DP cells in the induction of intestinal inflammation. A total of 1×10^6 Th2 (*A* and *E*) and Th1 (*B* and *F*) cells were transferred to *RAG-1*−/− mice. Four weeks later, the intestines were harvested and fixed in periodate fixing buffer. Tissues were embedded in paraffin blocks and sectioned. H&E-stained sections were examined for inflammation. (*C* and *G*) $CD4^+CD8\alpha\alpha^+$ DP cells were sorted from mice previously reconstituted with Th2 cells, and 1×10^6 cells were transferred to *RAG-1*−/− mice. Four days later, they were challenged with 3×10^6 Th1 cells. (*D* and *H*) Cells that were unable to produce IL-10 lacked protective ability. After 4 weeks, the intestines were harvested and sections were subjected to H&E (×100). Four animals were used per group, and the experiments were repeated twice with similar results. Histologic data shown are representative of two independent experiments.

IL-10 Is Not Required for the Phenotypic Development of $CD4^+CD8\alpha\alpha^+$ T Cells but Is Essential for Their Function. Our experiments show that DP iIELs can produce IL-10, and they can protect animals from Th1-induced intestinal inflammation. The observations that Tr1 cells can be generated by culturing $CD4^+$ T cells in an IL-10-containing medium (9) and that IL-10-deficient mice display spontaneous intestinal inflammation (27), led us to examine whether IL-10 plays a key role in the acquisition of $CD8\alpha$ molecules on $CD4^+$ T cells found in the intestinal epithelium. We first examined the abundance of DP cells in the intestine of IL-10−/− mice. We found that these mice have an increased number of DP

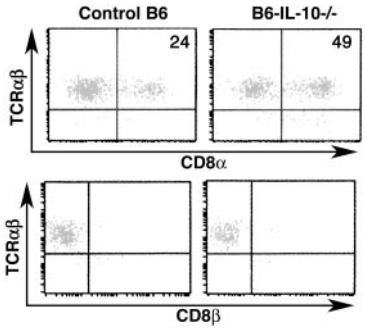

Fig. 5. $CD4^+CD8\alpha\alpha^+$ DP cells are found in the iIELs in IL-10−/− mice. iIELs were harvested from IL-10−/− mice and their control littermates. Cells were stained for their expression of CD8α. Gating on CD4 shows that IL-10−/− and its control littermates both contain $CD4^+CD8\alpha\alpha^+$ DP cells.

cells when compared with wild-type mice (Fig. 5). This suggests that whereas IL-10 can induce the Tr1 phenotype, IL-10 is not critical for the altered cell surface phenotype from CD4 into $CD4^+CD8\alpha\alpha^+$ DP T cells. To determine the role of IL-10 secreted by DP cells in the prevention of intestinal inflammation, we sorted DP cells from IL-10−/− mice, adoptively transferred them to syngeneic *RAG-1*−/− mice, and then challenged the recipient mice with Th1-polarized cells. As shown in Fig. 4 *D* and *H*, IL-10-deficient DP cells could not protect the animals from Th1-induced intestinal inflammation. Therefore, IL-10 produced by the DP cells plays a critical role in the prevention of intestinal inflammation.

$CD4^+$ T Cells from NF-κB-p50-Deficient and GATA-3DN Mice Are Deficient in IL-10 Production and Induce Intestinal Inflammation. We have shown that NF-κB p50−/− and GATA-3DN mice are unable to generate $CD4^+CD8\alpha\alpha^+$ DP cells in the intestinal epithelium. If DP cells are indeed important for the prevention of inflammation within the gut, we expected Th2 cells from these mice to lack the ability to prevent inflammation and instead contribute to, or at least to not inhibit, inflammation. To test these possibilities, we grew splenic $CD4^+$ T cells from these mice under Th2 culture conditions and transferred them to syngeneic *RAG-1*−/− and *RAG-2*−/− mice. As expected, these mice developed severe intestinal inflammation (Fig. 6*B*). Because our results suggested that IL-10 is critically involved in controlling inflammation, we expected $CD4^+$ T cells derived from p50−/− and GATA-3 DN mice grown under Th2 conditions to be deficient in IL-10 production. As shown in Fig. 6*A*, $CD4^+$ T cells isolated from such mice and grown under Th2 conditions produced dramatically reduced amounts of IL-10 when compared with their wild-type littermates. Thus, GATA-3 DN and NF-κB-deficient mice lack the ability to generate $CD4^+CD8\alpha\alpha^+$ DP IL-10-secreting cells and therefore cannot prevent the induction of intestinal inflammation. It is likely that a low level of IFN-γ produced in these cultures is responsible for the induction of colitis by these cells.

Discussion

IBDs are associated with chronic inflammation whose etiology is unknown. Cytokines such as IL-10 and TGF-β have been previously associated with inhibition of inflammation in the gastrointestinal tract (9, 28, 29). $CD4^+CD25^+$ regulatory T cells and B cells have been shown to regulate intestinal inflammation in an IL-10-dependent fashion (29, 30). Studies of IL-2/IL-2 receptor mutant mice, which are prone to IBD and have impaired intestinal T lymphopoiesis, first suggested that the iIELs may play a role in the regulation of intestinal inflammation (31). We report the identification of $CD4^+CD8\alpha\alpha^+$ DB cells present in the iIEL population that can suppress Th1 cell-induced intestinal inflammation in an IL-10-dependent fashion. These cells *in vivo* resemble the previously described Tr1 cells that were generated on *in vitro* culture with IL-10 (9).

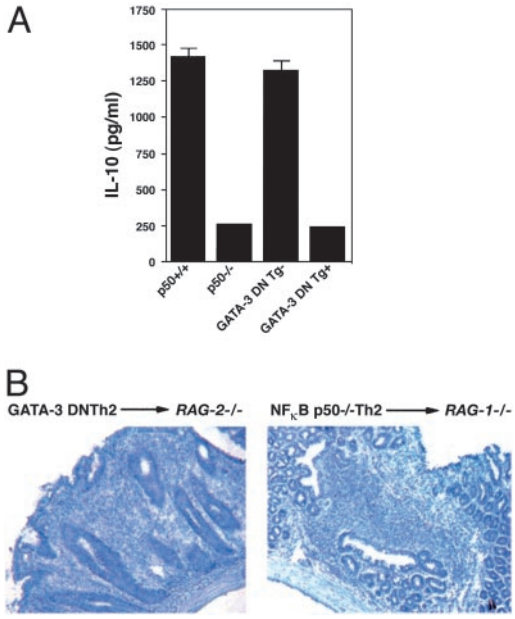

Fig. 6. GATA-3 DN and NF-κB p50−/− CD4 T cells produce less IL-10 and can induce intestinal inflammation. (*A*) CD4 T cells from GATA-3 DN or NF-κB p50−/− mice were cultured under Th2 polarizing conditions, and IL-10 secreted in the culture supernatant was measured by ELISA. (*B*) Th2 cells derived from CD4 T cells isolated from GATA-3 DN and NF-κB p50−/− mice were adoptively transferred to syngeneic *RAG-2*−/− and *RAG-1*−/− mice. Four weeks later, the intestines were harvested, fixed, and paraffin-embedded, and sections were stained with H&E and scored for inflammation (×100).

In our studies, the DP cells were isolated from the small intestine. Recently, two other papers have been published that show a role for the small intestine in protection against inflammation in the colon. In one report, another IEL subtype, $TCR\alpha\beta^+CD4^-CD8\alpha^+$ T cells, present in the small intestine and which were also shown to depend on IL-10 production for protection (the source being unknown), inhibited experimentally induced colitis (32). In the second paper, IL-10 produced from the small intestinal epithelium (using Tg mice expressing IL-10 specifically in the small intestine) prevented inflammation in the colon (33). There is no adequate explanation yet for how IL-10 produced in the small intestine confers protection on the large intestine. However, it is plausible that the DP cells induce increased IL-10 production from other cell types (enterocytes) or on stimulation by the local microenvironment are induced to secrete more IL-10 in the small intestine. Because IL-10 is an extremely potent immunosuppressive cytokine, the low levels reaching the large intestine are sufficient to block inflammation. In this regard, it will be interesting to know whether the intestinal inflammation initiates from the distal part of the small intestine and thus IL-10 production in the small intestine blocks the initiation of intestinal inflammation. Also, recently, it has been shown that Treg cells induce contact-dependent induction of contact-independent IL-10-producing regulatory $CD4^+$ T cells. These regulatory cells are then capable of influencing neighboring $CD4^+$ T cells (34). Therefore, it is possible that T cells from the periphery migrate to the small intestine first and on contact with Treg DP cells, they are induced to become regulatory T cells, which subsequently migrate to the large intestine.

Our results, as well as those of Poussier *et al.* (32), underscore the importance of iIELs in the maintenance of intestinal integrity (32). Both the DP cells as well as the SP $CD4^-CD8\alpha^+$ cells express the $CD8\alpha\alpha$ coreceptor. Whether the $CD8\alpha\alpha$ coreceptor plays an important role in inhibiting inflammation by the DP or $CD4^-CD8$

α^+ cells requires further investigation. An additional important finding in our studies is that a precursor $CD4^+$ T cell may be induced to become a DP regulatory cell on limited stimulation with IL-4 and exposure to the intestinal microenvironment or is irreversibly committed to a Th2 proinflammatory cell after repeated stimulation with IL-4. This suggests that dysregulation in intermediate steps in the development of these cells may tip the balance toward inflammation and may be one potential mechanism underlying the induction of inflammation within the intestines.

In contrast to conventional Th1 or Th2 cells, the primary cytokines produced by Treg cells are IL-10 and TGF-β. This distinct profile of cytokine secretion could be due to the influence of the microenvironment during their differentiation from precursor cells or they could be induced in Th2 cells after their migration into the intestinal epithelial layer. Recent studies show that Treg/Ts cells can be grown in the presence of IL-10 (9), a cytokine with immunosuppressive effects. IL-10-treated Th2 but not Th1 cells produce increased amounts of IL-10 (G.D., unpublished results). Several cell types, including activated macrophages, dendritic cells, B cells, and even epithelial cells, produce IL-10. The intestinal epithelium is continuously exposed to environmental antigens, particularly to the normal flora and to food antigens, which have the ability to activate epithelial cells or resident macrophages to produce IL-10. As a result, these epithelial DP mature T cells may be redirected to secrete IL-10, which is the property of typical Treg/Ts cells. Although we have tested several cytokines, including IL-4, IL-15, IL-10, TNF-α, and TGF-β, alone or in combination, we have been unable to identify the particular cytokine(s) that might influence Th2 cells to express the CD8α molecule on their surface. This could be because the intestinal epithelium has a distinct cytokine milieu, a possibility strengthened by our observation that Th2 cells that migrate to the spleen do not acquire CD8α (data not shown). Acquisition of CD8α could also result from close contact with intestinal epithelial cells and nonclassical MHC molecules.

Our studies show a previously unknown plasticity in GATA-3 function in T cells. Although GATA-3 plays an obligatory role in Th2 development, it inhibits the development of Th1 cells. Interestingly, two previous reports illustrate the importance of the level of GATA-3 expression in IFN-γ gene expression. Although one study showed that GATA-3 inhibits IFN-γ production in developing but not committed Th1 cells (35), a subsequent study showed that if a high enough level of GATA-3 is reached by forced expression, IFN-γ production can be also inhibited in committed Th1 cells (36). Collectively, these studies show that, at relatively low levels of GATA-3 expression, IFN-γ production can be sustained in T cells. Our studies add to these findings by showing that acquisition of CD8α and IL-10 production in the intestinal epithelial layer both depend on GATA-3 expression. It is unknown which factors are responsible for IFN-γ production in these cells. However, because recent studies have shown that the transcription factor T-bet is not essential for IFN-γ gene expression in $CD8^+$ T cells (37), it is possible that factors other than T-bet regulate IFN-γ production in the DP cells. The precise mechanisms underlying CD8$\alpha\alpha$ expression, IL-10, and IFN-γ gene expression have yet to be elucidated. Also, it is unclear from our adoptive transfer studies whether the DP cells were generated from developing Th2 cells or from an uncommitted precursor pool; we do not know whether these cells actually secreted IL-4 and then turned it off. However, even if the generation of DP cells involves distinct precursors, it is important to note that the development of both Th2 and DP cells depends on NF-κB and GATA-3.

Interestingly, it was found that only 30% of the $CD4^+$ T cells found in the intestinal epithelial layer have CD8α molecules. Similarly, in our Th2 transfer model, we also found that only 20–30% of the cells acquire the CD8$\alpha\alpha$ homodimer, and under no circumstances did this number increase. This could be explained in two ways. Either these cells represent a distinct repertoire of cells, or that after acquisition of CD8$\alpha\alpha$ on a certain number of Th2 cells equilibrium between Th2 and Treg cells is maintained. We do not support the former, because Th2 cells from AND TCR Tg mice also displayed similar characteristics. It is possible that after their generation, once the DP cells begin to produce high amounts of IL-10 in combination with IFN-γ, they shut off gene expression in other neighboring $CD4^+$ T cells. It should be noted that IL-10 has been shown to inhibit the expression of several genes including that of Th1 cytokine genes. IFN-γ has been also shown to inhibit the growth of Th2 cells directly and indirectly. It will be interesting to determine which occurs first, IFN-γ production or CD8α acquisition.

In summary, we have shown that $CD4^+CD8\alpha\alpha^+$ DP cells in the intestinal epithelial layer have important suppressive qualities. Our data show a mechanism for the generation of Ts/Treg cells in the intestinal epithelium that may be important in controlling IBD.

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1. Mombaerts, P., Mizoguchi, E., Grusby, M. J., Glimcher, L. H., Bhan, A. K. & Tonegawa, S. (1993) *Cell* **75,** 274–282.
2. Powrie, F., Correa-Oliveira, R., Mauze, S. & Coffman, R. L. (1994) *J. Exp. Med.* **179,** 589–600.
3. Simpson, S. J., Shah, S., Comiskey, M., de Jong, Y. P., Wang, B., Mizoguchi, E., Bhan, A. K. & Terhorst, C. (1998) *J. Exp. Med.* **187,** 1225–1234.
4. Bhan, A. K., Mizoguchi, E., Smith, R. N. & Mizoguchi, A. (1999) *Immunol. Rev.* **169,** 195–207.
5. Iijima, H., Takahashi, I., Kishi, D., Kim, J. K., Kawano, S., Hori, M. & Kiyono, H. (1999) *J. Exp. Med.* **190,** 607–615.
6. Dohi, T., Fujihashi, K., Rennert, P. D., Iwatani, K., Kiyono, H. & McGhee, J. R. (1999) *J. Exp. Med.* **189,** 1169–1180.
7. Boirivant, M., Fuss, I. J., Chu, A. & Strober, W. (1998) *J. Exp. Med.* **188,** 1929–1939.
8. Fort, M., Lesley, R., Davidson, N., Menon, S., Brombacher, F., Leach, M. & Rennick, D. (2001) *J. Immunol.* **166,** 2793–2800.
9. Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. & Roncarolo, M. G. (1997) *Nature* **389,** 737–742.
10. Kaye, J., Hsu, M. L., Sauron, M. E., Jameson, S. C., Gascoigne, N. R. & Hedrick, S. M. (1989) *Nature* **341,** 746–749.
11. Zhang, D. H., Yang, L., Cohn, L., Parkyn, L., Homer, R., Ray, P. & Ray, A. (1999) *Immunity* **11,** 473–482.
12. Das, G., Gould, D. S., Augustine, M. M., Fragoso, G., Sciutto, E., Stroynowski, I., Van Kaer, L., Schust, D. J., Ploegh, H., Janeway, C. A., Jr., & Scitto, E. (2000) *J. Exp. Med.* **192,** 1521–1528.
13. Berrih, S., Gaud, C., Bach, M. A., Le Brigand, H., Binet, J. P. & Bach, J. F. (1981) *Clin. Exp. Immunol.* **45,** 1–8.
14. De Maria, A., Malnati, M., Moretta, A., Pende, D., Bottino, C., Casorati, G., Cottafava, F., Melioli, G., Mingari, M. C., Migone, N., *et al*. (1987) *Eur. J. Immunol.* **17,** 1815–1819.
15. Ottenhoff, T. H., Elferink, D. G., Klatser, P. R. & de Vries, R. R. (1986) *Nature* **322,** 462–464.
16. Rocha, B., Vassalli, P. & Guy-Grand, D. (1994) *J. Exp. Med.* **180,** 681–686.
17. Reimann, J. & Rudolphi, A. (1995) *Eur. J. Immunol.* **25,** 1580–1588.
18. Morrissey, P. J., Charrier, K., Horovitz, D. A., Fletcher, F. A. & Watson, J. D. (1995) *J. Immunol.* **154,** 2678–2686.
19. Landry, D. B., Engel, J. D. & Sen, R. (1993) *J. Exp. Med.* **178,** 941–949.
20. Zhang, D. H., Cohn, L., Ray, P., Bottomly, K. & Ray, A. (1997) *J. Biol. Chem.* **272,** 21597–21603.
21. Das, J., Chen, C. H., Yang, L., Cohn, L., Ray, P. & Ray, A. (2001) *Nat. Immunol.* **2,** 45–50.
22. Sha, W. C., Liou, H. C., Tuomanen, E. I. & Baltimore, D. (1995) *Cell* **80,** 321–330.
23. Fujihashi, K., Yamamoto, M., McGhee, J. R., Beagley, K. W. & Kiyono, H. (1993) *Int. Immunol.* **5,** 1473–1481.
24. Nakamura, T., Lee, R. K., Nam, S. Y., Al-Ramadi, B. K., Koni, P. A., Bottomly, K., Podack, E. R. & Flavell, R. A. (1997) *J. Immunol.* **158,** 2090–2098.
25. Iqbal, N., Oliver, J. R., Wagner, F. H., Lazenby, A. S., Elson, C. O. & Weaver, C. T. (2002) *J. Exp. Med.* **195,** 71–84.
26. McDevitt, M. A., Shivdasani, R. A., Fujiwara, Y., Yang, H. & Orkin, S. H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6781–6785.
27. Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. & Muller, W. (1993) *Cell* **75,** 263–274.
28. Powrie, F., Carlino, J., Leach, M. W., Mauze, S. & Coffman, R. L. (1996) *J. Exp. Med.* **183,** 2669–2674.
29. Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. (1999) *J. Exp. Med.* **190,** 995–1004.
30. Mizoguchi, A., Mizoguchi, E., Takedatsu, H., Blumberg, R. S. & Bhan, A. K. (2002) *Immunity* **16,** 219–230.
31. Poussier, P., Ning, T., Chen, J., Banerjee, D. & Julius, M. (2000) *Gastroenterology* **118,** 880–891.
32. Poussier, P., Ning, T., Banerjee, D. & Julius, M. (2002) *J. Exp. Med.* **195,** 1491–1497.
33. De Winter, H., Elewaut, D., Turovskaya, O., Huflejt, M., Shimeld, C., Hagenbaugh, A., Binder, S., Takahashi, I., Kronenberg, M. & Cheroutre, H. (2002) *Gastroenterology* **122,** 1829–1841.
34. Dieckmann, D., Bruett, C. H., Ploettner, H., Lutz, M. B. & Schuler, G. (2002) *J. Exp. Med.* **196,** 247–253.
35. Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C. & Murphy, K. M. (1998) *Immunity* **9,** 745–755.
36. Lee, H. J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A. & Arai, N. (2000) *J. Exp. Med.* **192,** 105–115.
37. Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P. & Glimcher, L. H. (2002) *Science* **295,** 338–342.