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Expression of Interleukin-10 in Intestinal Lymphocytes Detected by an Interleukin-10 Reporter Knockin *tiger* Mouse

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Summary

To identify interleukin-10 (IL-10)-producing cells in vivo, we generated a knockin mouse where an internal ribosome entry site (IRES) green fluorescence protein (GFP) element was inserted immediately before the polyadenylation site of the IL-10 gene. GFP fluorescence in cells from these mice was found to correlate positively with IL-10 protein expression. With this model, we found that after multiple T cell receptor (TCR) stimulations, strong expression of IL-10 was produced specifically by intraepithelial lymphocytes (IEL) in the small intestine and colonic lamina propria lymphocytes (cLPL). We found that anti-CD3 treatment induces T regulatory cell 1 (Tr1)-like cells in small intestinal IEL (sIEL) and led to the accumulation of naturally occurring regulatory T (nTreg) cells in colonic LPL (cLPL). These findings highlight the intestine as a unique site for induction of IL-10-producing T cells, which play a critical role in the regulation of inflammation in the gut.

Introduction

Understanding how the immune system is controlled to avoid tissue destruction and other adverse effects caused by immunopathology associated with infection, autoimmune diseases, and allergy should provide an explanation for the etiology of such abnormalities and may lead to more effective treatment of disease.

One of the key molecules highly associated with immunological tolerance and suppression is interleukin-10 (IL-10). High serum IL-10 has been observed to correlate with remission of several diseases, especially those in which induction of immunological tolerance is critical. Understanding the mechanism by which IL-10 regulates the immune system might therefore yield a method for effective control of immunity by clinical intervention.

Because the most prominent phenotype observed in IL-10-deficient mice is inflammation in the intestine, IL-10 might be important in regulating inflammatory response in the intestine. Indeed, the importance of IL-10 in tolerance was demonstrated by the adoptive transfer model of inflammatory bowel disease into immunodeficient mice, which showed that IL-10 has a role in disease suppression (Asseman et al., 1999; Powrie et al., 1994). Given that intestinal organs have been reported to express IL-10 abundantly (Autschbach et al., 1998; Braunstein et al., 1997), it is necessary to analyze which cells express IL-10 in mucosal tissue. Such information might in turn lead to the understanding of the immunological regulation in intestine.

In the literature, a wide variety of cells have been reported to express IL-10 in vitro, including T helper 2 (Th2) cells, B cells, macrophages, and NKT cells, Th3 cells, $CD4^+CD25^+$ naturally occurring regulatory T cells (nTreg), T regulatory cell 1 (Tr1) (reviewed in Moore et al., 2001), and IEL (Das et al., 2003). Similarly, receptors for IL-10, IL-10R1, and IL-10R2 are also expressed on many cell types (Moore et al., 2001) and are often observed in IL-10-expressing cells, which suggests that the IL-10 producers themselves can also be targets. The multitude of possible IL-10 producers as well as targets underscores the importance of identifying which cells produce substantial amounts of this cytokine and which cells receive the signal in vivo.

Unfortunately, IL-10 detection is difficult, especially in vivo, because of its short half-life and rapid secretion. Although it is possible to stimulate cells ex vivo and detect the cytokine in supernatants or to analyze IL-10 intracellular staining by flow cytometry, it is not simple to correlate which positively stained cells provide the secreted IL-10. Furthermore, the signals obtained by intracellular staining are often too weak to identify the IL-10-producing cells in vivo.

Here, we show that the green fluorescence protein (GFP) knockin mouse, designated *tiger* (interleukin-ten ires gfp-enhanced reporter), provides a valuable tool for IL-10 analysis and that multiple rounds of TCR stimulation generated cells highly committed to produce IL-10 in vivo. Interestingly, these cells could be found preferentially in the intestine, in particular, IEL in the small intestine and LPL in the colon. The findings presented here implicate the intestine as a unique microenvironment for the development of and stimulus for IL-10-producing cells in vivo. By combining our previously described forkhead box P3 (Foxp3) knockin mice with the *tiger* mice, we characterized the relationship of Foxp3 and IL-10 expression in the intestine and analyzed IL-10-GFP expression in models of infection.

Results

Generation of *tiger* Mice

We generated a GFP knockin targeting vector (Figure 1A, middle) designed to undergo integration into the *Il10* locus (Figure 1A, top) by homologous recombination. The neo resistant gene was inserted at the end of the last exon not affecting the 3′-untranslated region. After successful electroporation and integration of the construct into embryonic stem (ES) cells, Cre-mediated excision of neomycin was performed to yield the mutant allele (Figure 1A, bottom) by direct transfection of Cre

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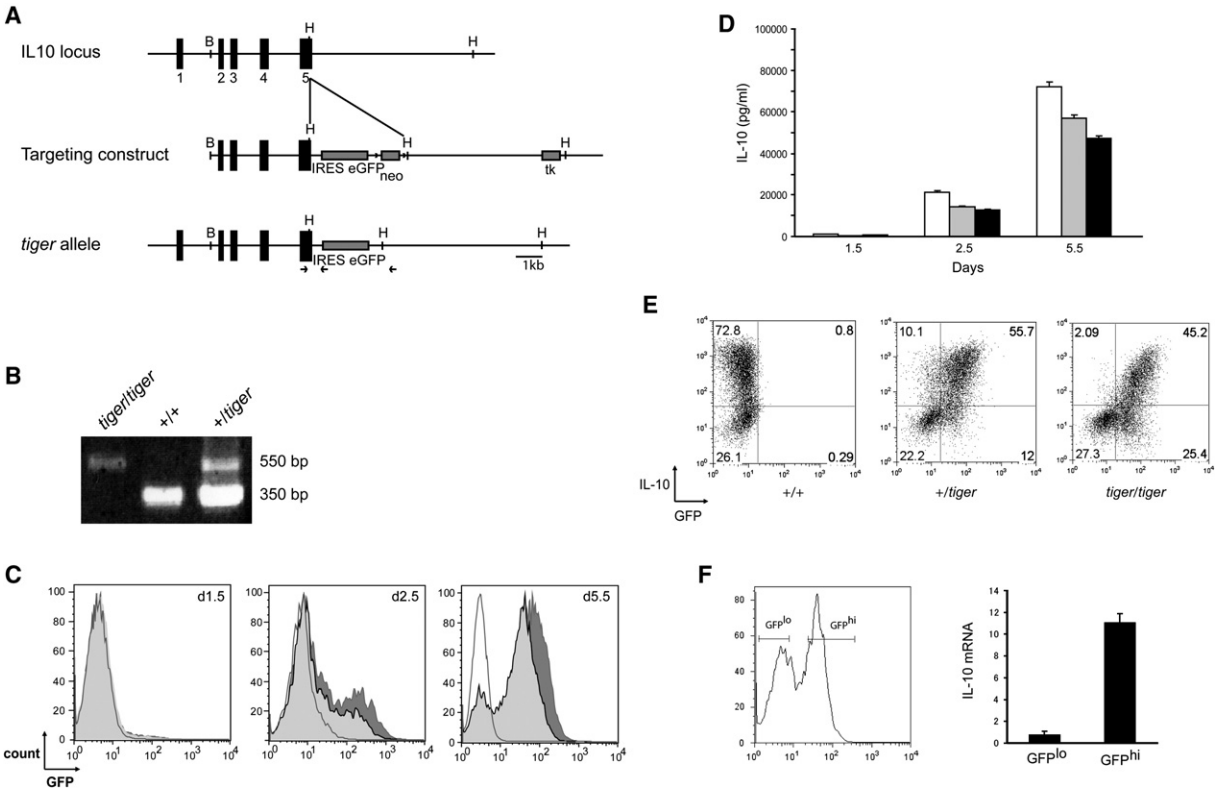

Figure 1. Generation of *tiger* Mouse and Expression Analysis In Vitro

(A) A 9.6 kb genomic fragment of the IL10 locus (top) was used to create the targeting vector (middle), into which an IRES-GFP cassette followed by a LoxP-flanked neomycin resistance gene was inserted between the stop codon and polyadenylation signal of exon 5. Deletion of neo by Cre recombinase resulted in the final mutant allele (bottom). Abbreviations: tk, thymidine kinase; B, BamHI; H, HindIII.

(B) Genotyping of *tiger* mice by PCR.

(C) FACS histograms of GFP profile of Th2 cells from control (no fill, foreground), heterozygous (light gray fill, middle), and homozygous (dark gray fill, background) *tiger* mice.

(D) Supernatants from the cultures described in the preceding panel at the time points indicated without restimulation of cells. Open, gray, and closed boxes show the concentration of IL-10 in wild-type, heterozygous, and *tiger/tiger* homozygous Th2 cell supernatants, respectively. The error bars show the standard deviation.

(E) Dot plots of PE-anti-IL-10 (vertical axis) versus GFP (horizontal axis) intracellular staining obtained from wild-type (left), heterozygous (middle), and homozygous (right) mice.

(F) Quantitative analysis of mRNA in GFP^{hi} and GFP^{lo} population of Th2 cells. The Th2 cells were differentiated in vitro and were FACS sorted as shown at left. Total RNA from the GFP^{hi} cells and GFP^{lo} cells were obtained and analyzed for IL-10 mRNA by the quantitative PCR. The data shown are the typical results from more than three experiments. The error bars show the standard deviation.

recombinase into positively selected ES cell clones, which were then injected into C57BL/6 blastocysts. Chimeric males were bred to C57BL/6 mice, and subsequent generations were bred to hetero- or homozygosity as experiments warranted. The resulting mice were viable and fertile, showing no gross phenotypic defects. At no time did any mouse develop inflammatory bowel disease, confirming that endogenous regulation of IL10 production remained intact. We have designated these animals *tiger* mice.

GFP Expression of *tiger* Th2 Cells

In order to validate IL-10 production and GFP expression, $CD4^+$ splenocytes from C57BL/6 and heterozygous and homozygous *tiger* mice were stimulated under Th0, Th1, and Th2 conditions. We could detect neither GFP nor IL-10 production in cells developed under Th0 or Th1 conditions (fewer than 1% positive cells), so we focused on IL-10 and GFP expression in Th2 cells. 1.5, 2.5, and 5.5 days after initiation of Th2 cultures, cells and supernatants were analyzed by flow cytometry and enzyme-linked immunosorbent assay (ELISA). The histograms in Figure 1C show an increasingly robust and distinct GFP^+ population over time. Further, the amount of IL-10 cytokine produced, as measured by ELISA (Figure 1D), correlated with the percentage of GFP^+ cells. Finally, IL-10 from both hetero- and homozygous *tiger* mice was upregulated to comparable amounts of production at the same kinetics as that of control mice. Interestingly, the mean fluorescent intensity (MFI) of cells from heterozygous *tiger* mice was slightly less than that of their homozygous counterparts (MFI = 46.2 ± 2.4 versus 59.2 ± 0.9 for hetero and homo, respectively).

When we stained these cells for intracellular IL-10, we observed that most of the IL-10-producing cells were GFP^+ and that IL-10 protein positively correlated with GFP expression. In heterozygous *tiger* mice, there was a small percentage of IL-10-positive, GFP-negative cells (Figure 1E, middle), whereas the corresponding population is virtually absent in homozygous mice (Figure 1E,

right). This result suggests that in this minority cell population in heterozygous mice, IL-10 was possibly transcribed off the wild-type but not the *tiger* allele. More importantly, the absence of IL-10-positive, GFP-negative cells in homozygous *tiger* mice indicates that all IL-10 production is coincident with GFP fluorescence. However, the fact that more than 50% of Th2 cells in heterozygous mice are double positive for IL-10 and GFP also suggests that both alleles are transcribed, arguing against monoallelic transcription of this cytokine.

To confirm that GFP expression reflected the IL-10 mRNA expression, we sorted the GFP^{hi} cells and GFP^{lo} cells from Th2 cells differentiated in vitro (Figure 1F). Quantitative PCR results showed more than a 10-fold difference of IL-10 mRNA expression between GFP^{hi} and GFP^{lo} cells. This result indicates that, by detecting GFP expression in cells of *tiger* mice, we can approximate the amount of IL-10 mRNA and that this system is useful as a reporter for IL-10 mRNA production.

GFP Expression in Macrophages and Dendritic Cells

It has been reported that IL-10 is expressed by macrophages (Kambayashi et al., 1996) and certain dendritic cell subsets (Akbari et al., 2001; Iwasaki and Kelsall, 1999). We therefore attempted to detect GFP expression in these cell types from the *tiger* mouse. In bone marrow-derived macrophages (BMDM), low basal GFP fluorescence could be seen when cells from *tiger* mice were cultured in medium alone (Figure 2A, MFI = 3.7 ± 0.4). However, when these macrophages were incubated in the presence of lipopolysaccharide (LPS) (Figure 2B, MFI = 8.2 ± 1.2), GFP expression was clearly elevated. Addition of IL-4 further increased GFP expression in macrophages (Figure 2C, MFI = 13.2 ± 0.3). The GFP expression correlated with the IL-10 produced in the culture supernatant (medium < 25 pg/ml, LPS 352 ± 20.4 pg/ml, LPS+IL-4 624 ± 10.7 pg/ml). This observation was consistent with previous findings by other groups (Kambayashi et al., 1996) and shows that GFP expression associates with conditions under which IL-10 is produced.

When we obtained BMDC and stimulated them with LPS, we could not detect any GFP expression (data not shown). Zymosan, a yeast cell wall component, potently induces IL-10 in dendritic cells (Rogers et al., 2005), so we stimulated BMDC with this reagent in the presence or absence of IL-4, IL-10, or transforming growth factor-β (TGF-β). As shown in Figure 2E, zymosan induced GFP expression in the *tiger* BMDC and enhanced it in the presence of TGF-β (Figure 2F, $p < 0.05$). Neither IL-4 nor IL-10 increased the GFP signal (data not shown). Intracellular staining of dendritic cells confirmed the upregulation of GFP expression in IL-10-expressing cells (Figures 2G and 2H) (although there is substantial autofluorescence of BMDC, we consistently observed upregulation of GFP in IL-10-positive cells in repeated experiments). However, in contrast to the result obtained with Th2 cells, the upregulation of the GFP in IL-10-producing cells was relatively low in BMDC. This result might suggest that either the *tiger* system is less sensitive in myeloid cells, perhaps because of the stability of GFP in these cells, or alternatively that the transcriptional regulation of dendritic cells and T cells might be different. Both dendritic cells and macrophages produce less

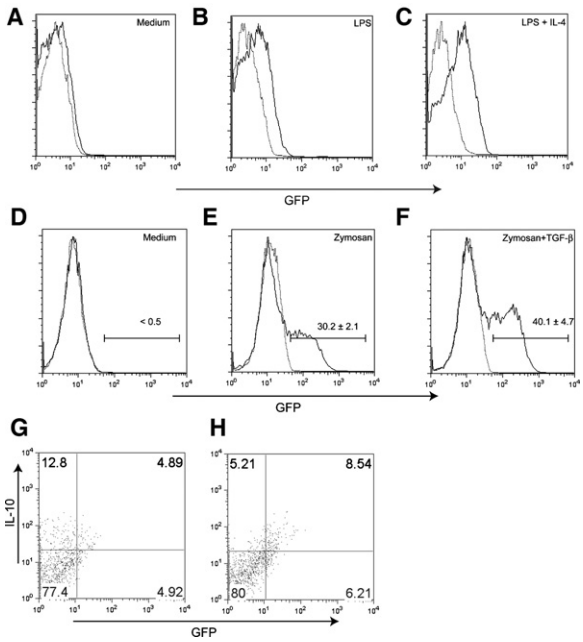

Figure 2. GFP Expression in Bone Marrow-Derived Macrophages and Dendritic Cells

(A–C) Macrophages derived from bone marrow cells were incubated with (A) medium alone, (B) LPS, or (C) IL-4 and LPS.

(D–F) BMDCs were cultured in the presence of (D) medium alone, (E) zymosan, or (F) zymosan and TGF-β. Dotted line represents GFP^- control cells and solid line represents cells from *tiger* mice. Mean and standard deviation (SD) is shown as "mean ± SD." Macrophages were gated for $CD11b^+$ and dendritic cells were gated for CD11c.

(G and H) Intracellular staining of BMDC with anti-IL-10 in (G) wild-type mice and (H) *tiger* mice are shown. The data shown are typical results of five experiments.

GFP and intracellular IL-10, so IL-10 transcription might be more strictly regulated. When we recovered dendritic cells ex vivo from spleen and Peyer's patches and stimulate them with zymosan, GFP expression was observed as only a slight shift of the histogram (see Figure S1 in the Supplemental Data available online), and there was no GFP high population. Thus, the expression of IL-10 in DC ex vivo was less than that of BMDC stimulated in vitro.

Taken together, we could confirm that a GFP signal can be induced in both macrophages and dendritic cells in response to stimuli that lead to IL-10 production.

Superantigen Stimulation Induces GFP in IEL and Peyer's Patch T Cells

To observe which cells express IL-10 in vivo, we analyzed GFP fluorescence in various cell types from untreated *tiger* mice. However, we could not find GFP^+ cells in unstimulated mice except for a slight increase in mesenteric lymph nodes (less than 3%) and colonic LPL (0%–7%) (see below). It is known that IL-10 is readily detectable in serum upon injection of mice with staphylococcal enterotoxin A (SEA), which stimulates a certain subset of T cells and subsequently anergizes them (Miller et al., 1999; Sundstedt et al., 1997); this finding is particularly visible upon repeated treatment of the Vβ expressing pigeon cytochrome C responsive TCR

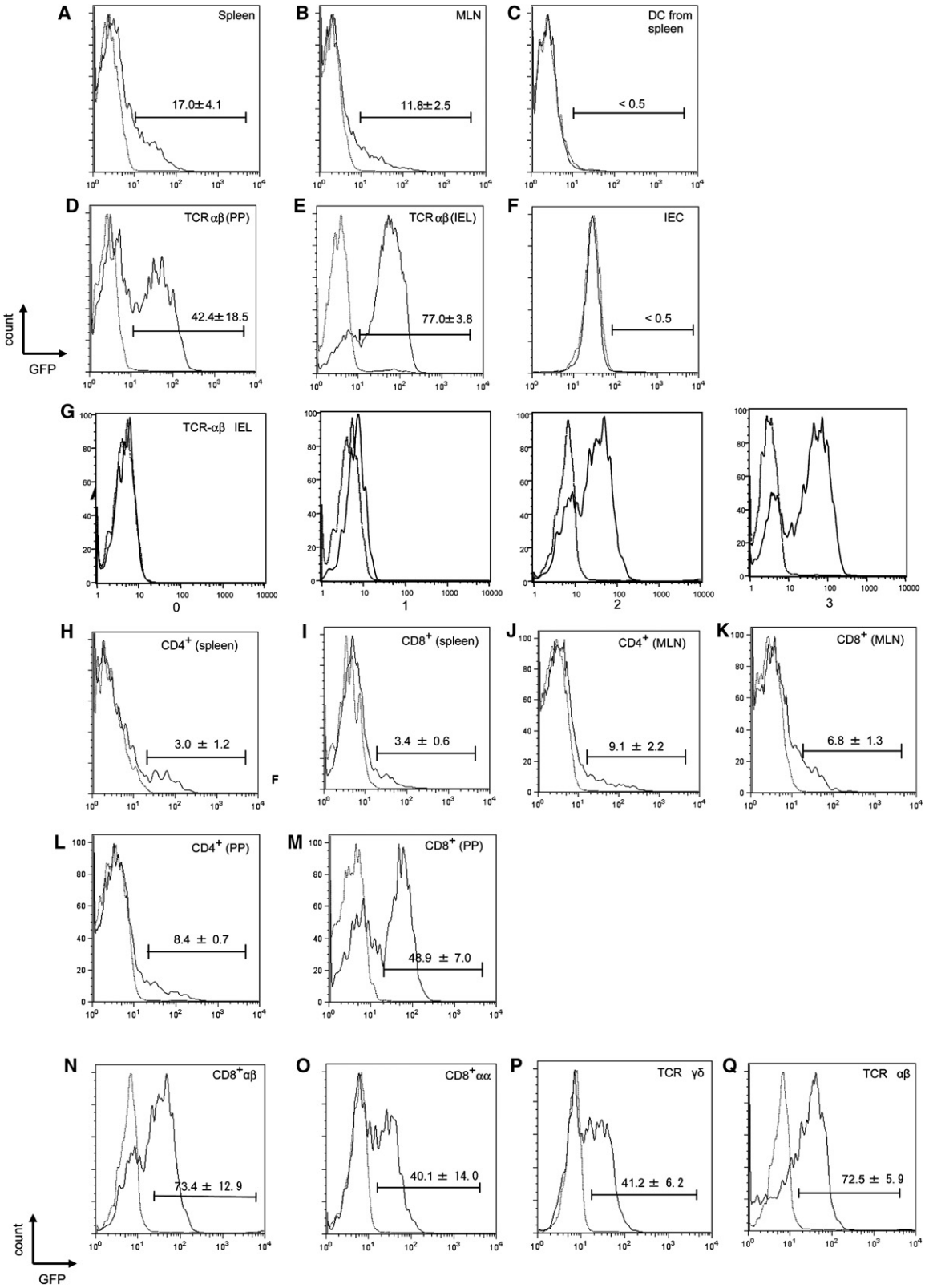

Figure 3. In Vivo Expression of GFP after Multiple Stimulation of TCR

tiger × AND TCR Tg mice (solid line) and the non-*tiger* AND TCR Tg mice (dotted line) were injected with SEA three times.

(A–F) 4 hr after final stimulation, TCR$\alpha\beta^+$ T cells from (A) spleen, (B) mesenteric lymph node, (C) CD11c$^+$ dendritic cells in spleen, (D) TCR$\alpha\beta^+$ lymphocytes in Peyer's patch, (E) TCR$\alpha\beta^+$ lymphocytes in IEL, and (F) intestinal epithelial cells in small intestine were recovered and expression of GFP was analyzed. Mean and SD is shown as "mean ± SD."

(G–Q) *tiger* mice (solid line) and the non-*tiger* mice (dotted line) were injected with 20 μg of anti-CD3 three times.

transgenic mice (AND) (Kaye et al., 1989). We therefore crossed *tiger* mice to AND TCR Tg mice. Littermates of *tiger* allele-negative, AND TCR transgene-positive mice were used as controls.

4 hr after injecting SEA, the GFP expression in cells from various organs was analyzed by flow cytometry. To our surprise, despite polyclonal activation in vivo, these mice showed a relatively low number of GFP^+ $CD4^+$ or $CD8^+$ cells from the spleen (Figure 3A) or lymph node (Figure 3B). However, a high percentage of IEL were positive for GFP (Figure 3E). In the same experiment, we examined dendritic cells from the spleen and intestinal epithelial cells, but we could not detect any GFP signal (Figures 3C and 3F, respectively).

It is also of interest that Peyer's patches contained a high number of GFP^+ T cells (Figure 3D) after SEA stimulation, whereas mesenteric lymph nodes contained far fewer GFP^+ T cells and in fact more closely resembled the pattern seen in the spleen (compare Figures 3B versus 3A). This result underscores the unique ability of the Peyer's patch to generate IL-10-expressing T cells.

To summarize, we found in this model of T cell tolerance that IL-10 was expressed at the highest amounts in IEL and Peyer's patch T cells.

Multiple In Vivo Stimulation with Anti-CD3 Induces GFP in IEL of *tiger* Mice

It has previously been shown that repeated in vivo treatment with anti-CD3 yields immune tolerance (Bach and Chatenoud, 2001). We therefore administered this antibody to non-TCR Tg *tiger* mice to enable detection of GFP under this tolerogenic protocol and to analyze exactly which cells were associated with this IL-10 expression. We obtained similar results with anti-CD3 stimulation to those obtained with SEA stimulation in TCR Tg mice (Figure 3G). Most IEL TCR$\alpha\beta$ cells were GFP^+ after three rounds of stimulation with anti-CD3. Interestingly, IEL increased their intensity of GFP signal as the frequency of injection increased (Figure 3G). In the same experiment, we examined $CD4^+$ and $CD8^+$ TCR$\alpha\beta^+$ T cells from spleen (Figures 3H and 3I, respectively) and mesenteric lymph nodes (Figures 3J and 3K). The expression pattern of GFP in these TCR$\alpha\beta$ cells was similar to the results obtained in *tiger* × AND TCR Tg mice; that is, they did not show a distinct GFP^+ population. In splenic T cells, we found a similar low frequency of GFP^+ cells in $CD4^+$ and $CD8^+$ T lymphocytes that we observed with SEA stimulation. This was not caused by a failure to activate splenic T cells, because most splenic T cells upregulated CD69 in response to the CD3 stimulus (Figure S3). In Peyer's patches, $CD4^+$ T cells expressed similar GFP expression to splenic T cells (Figures 3L versus 3H), but $CD8^+$ cells displayed high GFP expression reminiscent of those seen in stimulated IEL (Figures 3M versus 3G, 4th panel). Thus, based on our results, it appears that Peyer's patch $CD8^+$ cells have a greater potential to become IL-10-producing cells than do $CD4^+$ T cells. However, since 83%–88% of the T cells in Peyer's patches are $CD4^+$ $CD8^-$ cells, the T cells in this organ as a whole resembled the spleen in terms of overall GFP expression pattern after anti-CD3 treatment, a result that differed from the observations seen in AND TCR Tg mice stimulated with SEA. It is difficult to interpret this difference unequivocally, but we believe it might reflect the different mode of stimulation in vivo by superantigen versus anti-CD3. Superantigen is known to bind to the TCR-β chain and MHC class II, where it mimics antigen presentation by APCs; anti-CD3, in contrast, triggers a TCR signal by binding to cells that express Fc receptors and thereby enable crosslinking of the TCR. The difference of the distribution of class II molecules and Fc receptor expression might be responsible for this difference in GFP expression, which may, for example, require the involvement of tolerogenic APCs in order for a T cell to produce IL-10. Indeed, when we stimulated normal mice with SEA several times, more than 40% of V$\beta3^+$$CD4^+$ T cells in the Peyer's patch were GFP^+. As we show below that the Fc binding ability is necessary for the induction of IL-10 expression, this too suggests the importance of the accessory cells binding to the anti-CD3.

IEL comprise mainly TCR$\alpha\beta$ cells and TCR$\gamma\delta$ cells and, within the TCR$\alpha\beta$ subset, CD8$\alpha\alpha$ and CD8$\alpha\beta$ cells (Hayday et al., 2001). When we viewed each population separately, we found that TCR$\alpha\beta$ cells were consistently more GFP^+ than $\gamma\delta$ cells (Figures 3Q and 3P, respectively). Intestinal CD8$\alpha\beta$ cells are more like conventional T cells, whereas CD8$\alpha\alpha$ cells reside selectively in the IEL compartment. When we compared these two types of IEL, the frequency of GFP^+ CD8$\alpha\beta$ cells was always higher than that of GFP^+ CD8$\alpha\alpha$ cells (Figures 3N and 3O, respectively). This result suggests that conventional T cells might possess a greater potential to produce IL-10 in the IEL compartment.

The high expression of GFP in IEL was not dependent on the site of anti-CD3 administration, because both intraperitoneal and retro-orbital intravenous injection gave similar results (data not shown). Taken together, IEL and certain populations of Peyer's patch cells specifically acquire GFP expression and, therefore, IL-10 competence, after multiple rounds of stimulation with anti-CD3.

GFP Expression in IEL and LPL in the Small Intestine and the Colon

The major subsets of lymphocytes in the intestine are IEL and LPL. Furthermore, the lymphocytes in the small intestine and colon seem to have different characteristics in each compartment (Hayday et al., 2001). To elucidate further the differences between each population, we collected IEL and LPL from the small intestine and colon to determine IL-10 expression under a variety of conditions. The majority of TCR$\alpha\beta^+$ IEL in the small intestine (sIEL) were $CD8^+$ cells (Figure 4A), whereas

(G) TCR$\alpha\beta$ IEL were recovered after each injection and detected for GFP expression. The number indicated below each panel shows the number of injections. 4 hr after the final injection, cells from various organs were recovered and analyzed for GFP expression by flow cytometry.
(H–Q) GFP expression after three anti-CD3 injections. The GFP expression in (H) $CD4^+$ T cells and (I) $CD8^+$ T cells from spleen; (J) $CD4^+$ and (K) $CD8^+$ T cells from mesenteric lymph node; and (L) $CD4^+$ and (M) $CD8^+$ T cells from Peyer's patch. Cells from IEL in small intestine were detected for GFP. (N) CD8$\alpha\beta$ cells, (O) CD8$\alpha\alpha$ cells, (P) TCR$\gamma\delta$ cells, and (Q) TCR$\alpha\beta$ were gated to detect GFP. Mean and SD is shown as "mean ± SD." The data shown are typical results of more than five experiments.

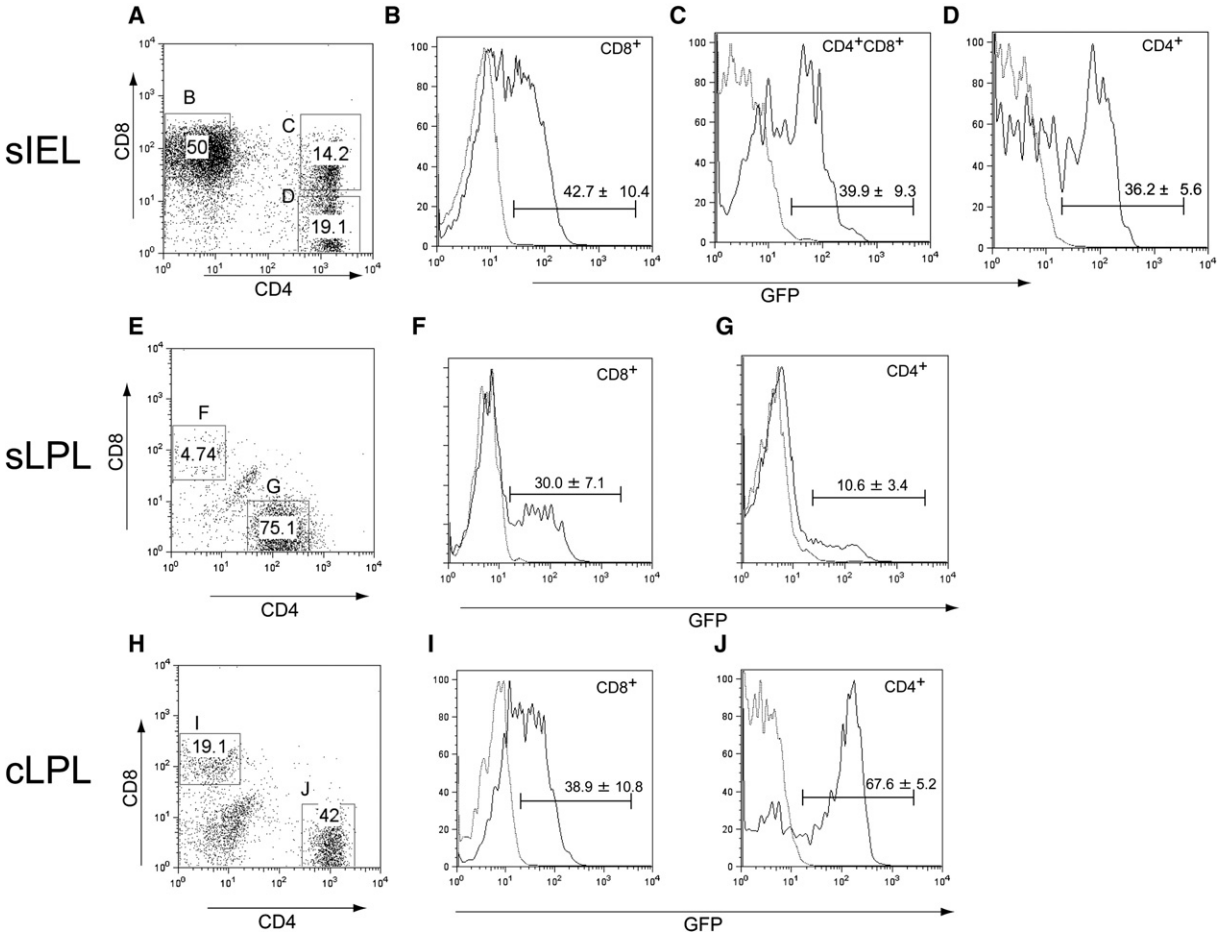

Figure 4. GFP Expression in IEL and LPL

tiger mice and control mice were injected with anti-CD3 three times. 4 hr after the last injection, mice were sacrificed and the GFP expression of IEL and LPL from small intestine and colon were analyzed.

(A, E, and H) Distribution of $CD4^+$ cells, $CD8^+$ cells, and $CD4^+CD8^+$ double-positive cells in $TCR\alpha\beta^+$ population in sIEL, sLPL, and cLPL, respectively. The gates shown in these panels were used for the following analysis of GFP expressions.

(B–D) GFP expression of (B) $CD8^+$, (C) CD4CD8 double-positive, (D) $CD4^+$ $TCR\alpha\beta^+$ IEL from small intestine.

(F and G) GFP expression of (F) $CD8^+$ and (G) $CD4^+$ $TCR\alpha\beta^+$ cells from LPL from small intestine.

(I and J) GFP expression of (I) $CD8^+$ and (J) $CD4^+$ LPL from colon. Mean and SD is shown as "mean ± SD." The data shown are typical results of three experiments

~75% of sLPL T cells were $CD4^+$ (Figure 4E) and the remainder $CD8^+$. In the small intestine, a high percentage of $CD8^+$ sIEL were GFP^+ as described above (Figure 4B). In contrast, GFP expression in $CD4^+$ T cells from the LPL population was much lower compared to that of IEL both in cell count and intensity (Figure 4G). $CD8^+$ cells and $CD4^+$ cells represent the major T cell population in IEL and LPL, respectively, so the comparison of IEL and LPL $TCR\alpha\beta$ T cells gives a very different pattern of GFP expression. When we repeated these experiments with sIEL and sLPL from the same mice at the same time point to eliminate variability (data not shown), we obtained the same results, confirming that this difference is not because of the lengthy procedure required to collect sLPL. In this experiment, minor populations such as $CD4^+$ cells in IEL and $CD8^+$ cells in LPL were also found to have various amounts of GFP^+ cells (Figures 4C, 4D, and 4F).

We also observed GFP expression in the large intestine after three in vivo stimulations with anti-CD3, but the cell types involved were different from that observed for the small intestine. Interestingly, a high frequency of $CD4^+$ cells, which represent the major population in LPL, were GFP^+ (Figure 4J). The minor $CD8^+$ T cell population was also GFP^+ in the LPL compartment (Figure 4I). However, GFP expression of the IEL from the colon was similar to the small intestine (data not shown). These results suggest that the colon is a distinct immunologic environment from the small intestine, especially with regard to LPL. GFP-expressing cells were rarely found in control or unstimulated mice (Figure 5K), so IL-10 expression is inducible, being upregulated in this case.

Anti-CD3 Induces Tr1-like Cells in sIEL- and Foxp3-Positive Cells in cLPL

To further characterize the IL-10-positive cells in the intestine, we examined the expression of other cytokines and Foxp3. First, to confirm that IL-10 protein expression correlated with GFP expression in IEL, we stained intracellular IL-10. As shown in Figure 5A, there was

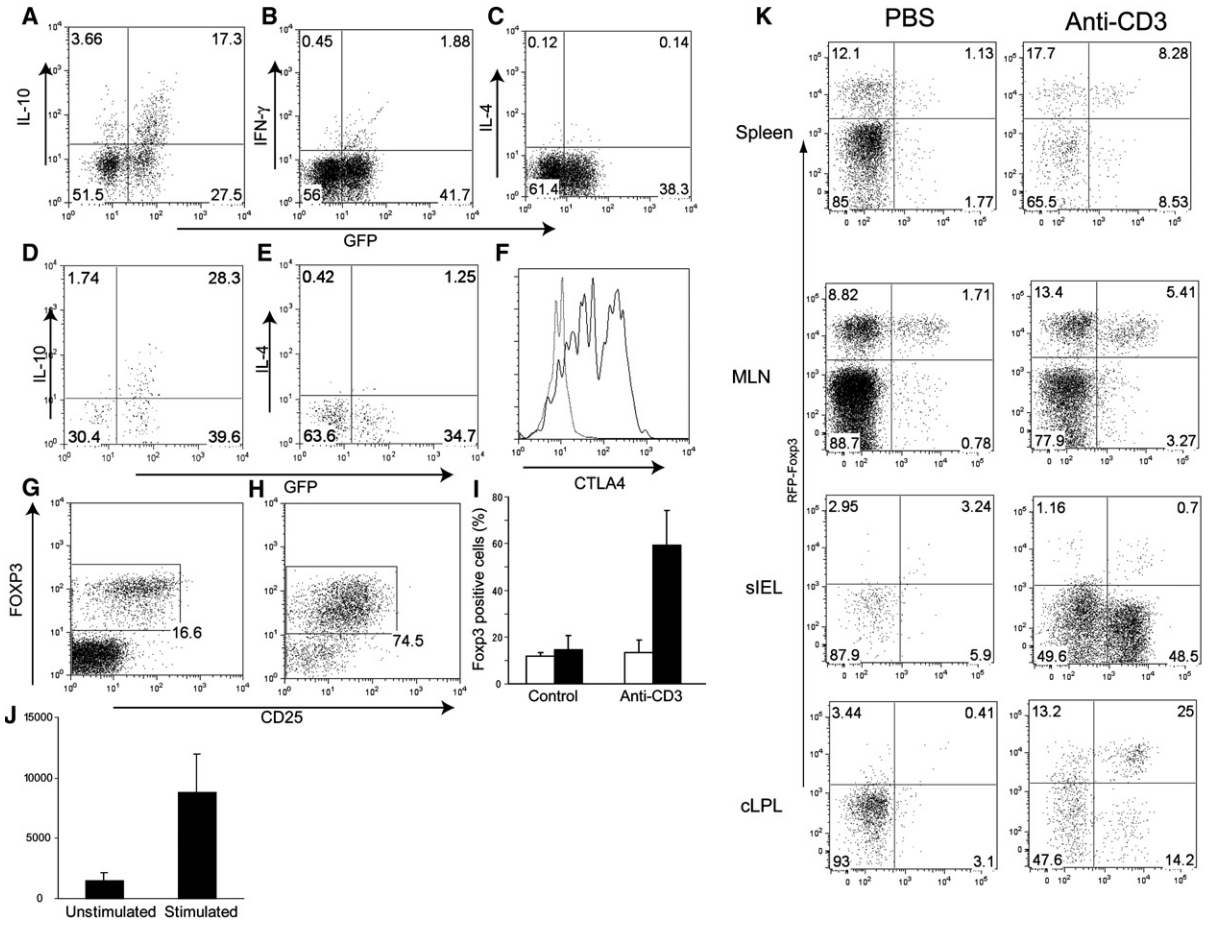

Figure 5. Characterization of sIEL and cLPL after Repetitive Anti-CD3 Treatment and Relationship of Foxp3 and IL-10 Analyzed in FIR × *tiger* Mice

After three anti-CD3 injections, TCR$\alpha\beta^+$ cell in sIEL (A–C) and cLPL (D–F) were collected and analyzed for (A, D) IL-10, (B) IFN-γ, and (C, E) IL-4. Horizontal axis shows the GFP expression of these cells. (F) CTLA-4 protein in CD4$^+$TCR$\alpha\beta^+$ cells detected by intracellular staining.

(G and H) After three anti-CD3 injections, (G) splenic CD4$^+$ cells and (H) colon CD4$^+$ LPL cells of TCR$\alpha\beta^+$ cells were stained with anti-CD25 and further stained intracellularly with anti-Foxp3 and detected with FACS.

(I) The percentage of Foxp3$^+$ cells within CD4$^+$ cells from cLPL and spleen in anti-CD3-treated and control (PBS-injected) mice. White bars represent the percentages of Foxp3$^+$ cells within CD4$^+$TCR$\alpha\beta^+$ cells in spleen cells, and black bars represent those in colonic LPL cells. The error bars show the standard deviation.

(J) The total number of Foxp3$^+$ CD4$^+$ T cells obtained from mice unstimulated and stimulated with anti-CD3. The data shown are representative of more than six similar results. The error bars show the standard deviation.

(K) FIR × *tiger* mice were treated with PBS (control) or anti-CD3 three times, and the cells were collected from spleen, mesenteric lymph nodes (MLN), IEL from the small intestine (sIEL), and LPL from the colon (cLPL). The cells from spleen, MesLN, sIEL, and cLPL are the gated population of TCR$\alpha\beta^+$CD4$^+$ cells and sIEL are gated for TCR$\alpha\beta^+$ cells. The data shown are representative of more than three similar experiments.

again a positive association of IL-10 production and GFP expression, similar to the results obtained with Th2 cells developed in vitro. In this experiment, we also stained for IFN-γ and IL-4, but there were few positive cells (Figures 5B and 5C), suggesting that these GFP$^+$ cells are not Th2- or Th1-like cells but more characteristic of Tr1 cells, which produce only IL-10. We stained intracellular foxp3 protein, but in IEL from the small intestine less than 1% of TCR$\alpha\beta^+$ cells were positive for Foxp3, and therefore do not have the characteristics of nTreg cells.

In colonic LPL cells, we also performed intracellular cytokine and Foxp3 detection. Similar to the results of IEL in small intestine, the IL-10 protein expression of colonic LPL were confirmed to correlate with GFP expression (Figure 5D), and IL-4 (Figure 5E) and other cytokines like IL-2 and IFN-γ (data not shown) were undetectable. Interestingly, these colonic LPL expressed CTLA4 after repetitive anti-CD3 stimulation (Figure 5F), and the number of colonic CD25$^+$ Foxp3-expressing cells increased (Figures 5H–5J), although there was no major change in the nTreg cell number in spleen after anti-CD3 treatment (Figures 5G and 5I). To determine whether cell proliferation contributes to this accumulation of nTreg cells in cLPL, we administered BrdU in the drinking water during the anti-CD3 treatment and stained the cLPL with BrdU antibody. CD4$^+$CD25$^+$ T cells in cLPL proliferated in contrast to splenic CD25$^+$ T cells, which did not (Figure S2). This result suggests that the increased number of Treg cells in cLPL is dependent on proliferation of nTreg cells, at least to some extent.

The high GFP expression in colonic LPL seemed to be associated with the accumulation of nTreg cells at that site, which strongly suggested that these nTreg cells

expressed high GFP and IL-10. To further elucidate the relationship between IL-10-expressing cells and Foxp3-expressing nTreg cells, we crossed the Foxp3-IRES-mRFP (FIR) reporter mouse (Wan and Flavell, 2005) and the tiger mouse. The results obtained from the cells from control and anti-CD3-stimulated mice are shown in Figure 5K. In spleen and mesenteric lymph nodes, less than 3% of the cells were positive for GFP in control mice, and both in Foxp3-expressing and -nonexpressing cells were GFP$^+$. After several injections of anti-CD3, we observed an increase of GFP positivity in both Foxp3$^+$ and Foxp3$^-$ cells.

In sIEL TCR$\alpha\beta^+$ cells, there were few Foxp3 cells, and the GFP$^+$ cells induced after anti-CD3 injection was mostly in the Foxp3$^-$ population, confirming that Tr1-like cells arise after anti-CD3 stimulation. In contrast, in cLPL cells, the number of Foxp3$^+$ cells increased after anti-CD3 treatment and the GFP-expressing cells were mainly observed in the RFP$^+$ (Foxp3$^+$) population. There were also a substantial number of RFP(Foxp3)-negative GFP$^+$ cells, suggesting that some Tr1-like cells arise in cLPL after anti-CD3 injection.

To summarize, repetitive anti-CD3 treatment induces Tr1-like cells in IEL of the small intestine, whereas the same treatment induces mostly nTreg cells in LPL in colon.

CD4$^+$ T Cells Adoptively Transferred to RAG-Deficient Mice Differentiate to IL-10-Expressing Cells in the Intestine

Our experiments suggested that IEL in the small intestine are committed to producing IL-10. This high IL-10 production from IEL might be acquired as a consequence of being a specific IEL lineage or may result from the ability of the gut surrounding the IEL to provide a special environment, causing them to differentiate into IL-10-producing cells.

To elucidate the importance of the gut environment for T cells to develop into IL-10-producing cells, we investigated whether conventional CD4$^+$ cells from the spleen can differentiate into IEL-like IL-10-producing cells when transferred into immunodeficient RAG knockout mice, which would lead to colonization of the gut. 2 months after transfer of splenic CD4$^+$ cells to RAG-deficient mice, we recovered splenocytes and IEL and scored the GFP signal by flow cytometry 4 hr after a single stimulation with anti-CD3. As shown in Figure 6D, splenic T cells did not express much GFP, whereas again, IEL had a higher percentage of GFP$^+$ cells (Figures 6B and 6C). It has been reported that CD4$^+$ cells enter the IEL compartment and acquire CD8 on their surface when transferred to RAG-deficient mice (Das et al., 2003). We were able to reproduce that observation (Figure 6A) and found that a high percentage of both CD4 single-positive (Figure 6B) and CD4$^+$CD8$^+$ double-positive (Figure 6D) cells were GFP$^+$, with a higher percentage of GFP$^+$ cells in the CD4$^+$CD8$^+$ double-positive population. It was noteworthy that only one injection of anti-CD3 was sufficient to induce measurable GFP expression, a strikingly different result from that which we obtained with the nontransfer model; i.e., the steady-state situation in the *tiger* mouse. To summarize, conventional T cells can differentiate into IL-10-expressing cells when transferred into immunodeficient hosts, but this occurs selectively in the intestinal environment.

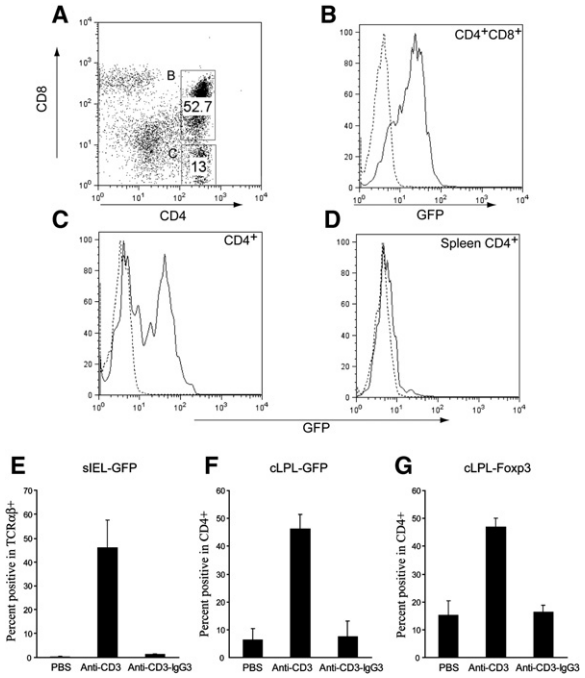

Figure 6. CD4$^+$ T Cells Acquire a GFP$^+$ IEL-like Phenotype when Transferred to RAG-Deficient Mice, and Mitogenic Ability of Anti-CD3 Is Required to Induce IL-10-Expressing IEL and cLPL

CD4$^+$ T cells were derived from *tiger* or control mice and adoptively transferred to RAG-deficient mice. 2 months after the transfer, mice were injected with anti-CD3 and sacrificed 4 hr after injection. The splenocytes and IEL from small intestine were collected and analyzed for GFP.

(A–C) CD4 versus CD8 staining profile of TCR$\alpha\beta^+$ IEL (A), and the gates used in (B) and (C). The GFP expression of (B) CD4$^+$CD8$^+$ double-positive IEL and (C) CD4$^+$ IEL are shown.

(D) GFP expression of CD4$^+$TCR$\alpha\beta^+$ T cells from spleen.

(E–G) Anti-CD3, anti-CD3-IgG3, or PBS was injected to the *tiger* mice and control mice three times every third day, and the IEL and LPL were corrected to detect GFP (E, F) and Foxp3 expression (G). Foxp3 was detected by intracellular staining followed by FACS detection. Percentage of GFP$^+$ cells in TCR$\alpha\beta^+$ cells in sIEL, CD4$^+$ cLPL, and Foxp3$^+$ from CD4$^+$ cLPL are shown. The experiments were repeated for three times with similar results. The error bars show the standard deviation.

Requirement of Fc Receptor Binding of Anti-CD3 to Generate IL-10-Producing T Cells in sIEL and cLPL

The generation of IL-10-expressing cells in the intestine might depend on the ability of the anti-CD3 to bind to other accessory cells and crosslink the TCR signaling component. To address this issue, we injected anti-CD3 engineered to lower the binding activity to Fc receptor (anti-CD3-IgG3) (Alegre et al., 1995). When we detected GFP$^+$ cells after three injections of anti-CD3-IgG3, there was no increase of GFP$^+$ cells in sIEL nor in cLPL (Figures 6E and 6F). Likewise, there was no increase of Foxp3$^+$ cells after the injection of this antibody (Figure 6G). Thus, the increase of GFP$^+$ cells by the injection of anti-CD3 depends on the Fc binding ability and mitogenic activity of this antibody.

Expression of GFP in the *tiger* Reporter Mouse after *Salmonella* and *Leishmania* Infection

The *tiger* system can be used to study IL-10 expression in a number of settings such as during microbial

infection. We therefore infected *tiger* reporter mice with *Salmonella typhimurium*, enteropathogenic bacteria that is known to induce profuse inflammation in the intestinal tract. 2 and 5 days after infection, mice were sacrificed, and the presence of GFP-producing cells in the intestine, spleen, and mesenteric lymphnodes were investigated as indicated in Experimental Procedures. 2 days after bacterial infection, no GFP-producing cells were detected among Peyer's patches cells, sIEL, cLPL, intestinal epithelial cells, splenic T or B cells, or macrophages. 5 days after infection, modest number of GFP-expressing cells were detected among cLPL, the majority of which were CD25$^+$ T cells (Figures 7A and 7B).

As a model of macrophage infection and the induction of IL-10-producing cells in parasitic infection, we used *L. major*. 28 days after footpad infection with *L. major*, the mice were sacrificed and examined for GFP expression. We first analyzed the draining popliteal lymph node and found only few GFP$^+$ T cells and no GFP$^+$ macrophages (Figure 7C). When we isolated lymphocytes from the inflamed footpad tissue, we could detect more GFP$^+$ CD4 T cells (Figure 7D). There appeared to be an increase in GFP expression in the previously GFP$^-$ cell population as well as a second population of GFP^{hi} cells (Figure 7D). More than 70% of these GFP^{hi} cells were CD25$^+$ by dot plot analysis, suggesting that these cells may be nTreg cells (data not shown). Macrophages and CD11c$^+$ cells from the infected tissue were again negative for GFP.

Discussion

The generation of the *tiger* mouse enables the identification of cells committed to produce IL-10. In this report, we have found this model to be useful in identifying cells producing IL-10, especially in vivo. The advantage of this reporter mouse is the ability to combine GFP analysis with multicolor staining with other antibodies, which enabled us to perform a detailed analysis of IL-10-producing populations.

Because the GFP gene was knocked in to the 3′ end of the IL-10 gene downstream of the 3′-UTR, its expression should reflect IL-10 mRNA expression. From our findings, GFP expression was positively associated with IL-10 protein expression. In the same experiments, we also found a small GFP-positive, IL-10-negative population. This difference may be due to a possible posttranscriptional or translational regulatory mechanism or because GFP is inherently more stable than IL-10 protein and may persist longer in cells. Because we find that the IL-10 amount is roughly proportional to GFP expression, we think that, in the *tiger* mouse, the GFP expression fairly reports the production of IL-10 and with higher sensitivity than intracellular staining. However, caution must be exerted in the interpretation of results with these mice, because essentially we report IL-10 mRNA and translational control of IL-10 mRNA will not be reported in this model. Furthermore, the result might differ depending on the location of insertion of the reporter gene within the IL-10 locus and whether an IRES sequence was used or not. Future use of our system might be helpful in identifying posttranscriptional regulatory mechanisms by staining for IL-10 together with GFP in combination with other stimuli that might potentially affect this posttranscriptional regulation.

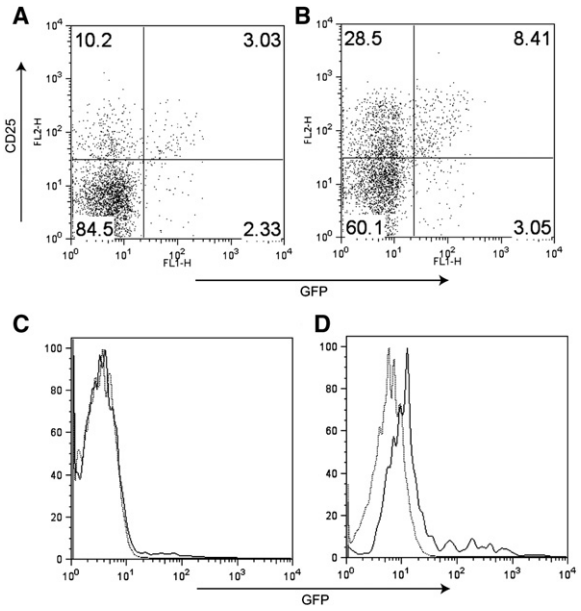

Figure 7. GFP Expression of T Cells after *S. typhimurium* and *L. major*

(A and B) GFP knockin mice were infected with 10^5 of *S. typhimurium* per oral and sacrificed after 5 days. The LPL from colon were prepared from (A) noninfected and (B) infected mice.

(C and D) The isolated cells were gated for CD4$^+$TCRβ^+ and plotted. The results obtained from *L. major* infection are shown in (C) and (D). GFP knockin mice or control mice were infected with *L. major* for 28 days. The CD4$^+$TCRβ^+ lymphocytes from (C) popliteal lymph nodes and (D) footpads were isolated and subjected to FACS analysis. Dotted line represents GFP$^-$ control cells and solid line represents cells from *tiger* mice. Experiments were repeated twice with 3–5 mice per group and similar results were obtained.

Our data show that IL-10 expression is not strictly monoallelic, because more than 60% of CD4$^+$ T cells were positive for GFP when cells were cultured under Th2 conditions. This observation is congruent with the results from the very similar 4GET IL-4 knockin mice, where more than 60% of the cells were positive for GFP under Th2 conditions (Mohrs et al., 2005).

After multiple rounds of TCR (CD3 antibody: 145-2C11) or superantigen stimulation in vivo, T cells produced IL-10 as revealed by increased GFP as well as IL-10 protein expression. This result suggests that T cells might differentiate into IL-10-producing cells after several cycles of TCR stimulation.

Stimulating T cells multiple times in vivo has been known previously to induce high IL-10 in serum, but the major source of IL-10 has never been clear (Sundstedt et al., 1997). It might logically have been expected that T cells in spleen and lymph nodes might be the major source, but our results suggest that gut IEL may be most important, particularly given the fact that the IEL are the largest T cell population in the body. It is possible that other disease models that also exhibit high serum IL-10 might have similarly high IL-10 expression by gut IEL. Further analysis is necessary to elucidate the relationship between the suppressive IL-10-expressing T cells induced by TCR stimuli and the intestinal lymphocytes.

From our measurements of Foxp3 and intracellular cytokine staining, it was suggested that sIEL and cLPL are fundamentally different cell types although they both

express IL-10 mRNA strongly and do not express other cytokines like IL-2, IL-4, and IFN-γ. One important question would be whether these IL-10-producing cells developed in situ and expanded in number locally or alternatively migrated from other sites and acquired IL-10 production. In the case of sIEL, both TCRαβ and γδ T cells were shown to be GFP$^+$ after stimulation, with the higher percentage of GFP$^+$ cells being TCRαβ cells. When we compared CD8αα and CD8αβ TCRαβ T cells, we found that a higher percentage of CD8αβ cells produced GFP. CD8αβ is characteristic of conventional T cells, so it might be that conventional T cells migrate from outside the IEL compartment and differentiate there into IL-10-producing cells. This idea is consistent with the fact that the profiles of CD4, CD8, and CD103 expression changes after anti-CD3 injection and showed an increased number of CD4$^+$ T cells and CD8αβCD103$^-$ T cells (M.K., R.A.F., unpublished data), which suggests the mobilization of conventional T cells into IEL compartment.

In the case of nTreg cells in cLPL, they might derive from existing nTreg in cLPL or be recruited from other tissue sites or peripheral blood. Because the number of Treg cells increased after TCR stimulation, the increased ratio of GFP$^+$ cells does not seem to be the result of the deletion of GFP$^-$ cells but rather proliferation of cLPL. It is, however, also possible that migrating cells from other sites might contribute to increase the number of nTreg cells in cLPL. Further work will be required to elucidate this.

As shown in our results, there was some variability in the frequency (40%–70%) and number of nTreg cells induced after anti-CD3 stimulation. This might be caused by genetic factors or environmental factors like commensal bacteria. However, we consistently observed induction of GFP$^+$ Treg cells in cLPL in more than eight experiments with littermates as controls for genetic variability.

From our observations, it appears that intestinal lymphocytes are strongly committed to IL-10 production, which is markedly different from what is seen with conventional T cells. Such a difference in phenotype might be a consequence of the special environment in the gut where specific factors may influence the differentiation of T cells. This possibility was confirmed by adoptive transfer of CD4$^+$ cells to RAG-deficient mice, which showed that conventional T cells can differentiate into IL-10-producing IEL-like cells but not into IL-10 producers in the spleen. The identity of the molecule(s) that are required for upregulation of IL-10 needs to be elucidated in future studies.

The repetitive administration of anti-CD3 has been found to be an effective therapy for type I diabetes (T1D) (Chatenoud et al., 1994) and graft-versus-host disease (GVHD) (Nicolls et al., 1993). The phenomenon that repetitive stimulation of anti-CD3 induces gut IEL cells to express IL-10 might not occur in patients treated with this therapy because the nonmitogenic CD3 antibody is now used for clinical trials in humans, and at least the murine nonmitogenic anti-CD3 did not induce Tr1-like cells in IEL nor nTreg cells in cLPL in *tiger* mice in our experiments. However, the antibodies used in the human trials probably retain residual Fc binding ability because some symptoms like fever and rash, which suggest mitogenic activity for this antibody, happens frequently in patients treated with this therapy. It is, therefore, still not completely ruled out that the nonmitogenic anti-CD3 might act on intestinal lymphocytes to induce suppressive cells.

We attempted to detect GFP positivity in cell types other than T cells, but we could not identify strong signals in macrophages, B cells, or dendritic cells in vivo. All these cells have been reported to produce IL-10 in vitro, but our reporter mouse might not be sensitive enough to detect low amounts of IL-10 production. Despite single or multiple rounds of stimulation with LPS (100 μg 3 times) in vivo, no induction of GFP was observed 6–24 hr poststimulation. The dose we used in vivo might not be enough to maintain the concentration used in vitro.

We could detect IL-10 production in myeloid cells in vitro. As shown in the in vitro culture experiments, however, the GFP expression of non-T cells is still not high as that of T cells. This might result from different IL-10 regulatory mechanisms in distinct cell types. Because the GFP expression relative to IL-10 staining was relatively low in dendritic cells, the mRNA expression of IL-10 might be low but translation of IL-10 might be more efficient per given mRNA molecule in DC than T cells. This is in line with the fact that the IL-10 protein expression is much less in myeloid cells compared to T cells.

The GFP expression of dendritic cells ex vivo was less than in vitro stimulated BMDC. We currently do not know why this is so. As reported by others (Edwards et al., 2002), additional stimuli such as CD40L might be necessary to detect IL-10 expression in dendritic cells derived ex vivo. Further analysis in the future will be necessary to study the mechanism underlying the differences of IL-10 production in T and non-T cells.

We infected *tiger* mice with *S. typhimurium* and *L. major* to examine IL-10 production during microbial infection. We found modest increase in the number of GFP-expressing cells 120 hr after inoculation, which is the time of maximal intestinal inflammation in this model of infection. This finding is in line with the notion that IL-10 can be induced during chronic inflammation. It is possible that the strong inflammatory response could result in the induction of nTreg cells in the inflamed colon as the GFP$^+$ cells in cLPL were mostly CD25$^+$ T cells.

Leishmania infection is a model of macrophage infection that leads to the induction of Th1 cells in C57BL/6 mice and Th2 cells in Balb/c mice, which are responsible for the resistance and susceptibility of these strains, respectively. During *Leishmania* infection of *tiger* mice, GFP induction was found in the T cells isolated from the inflammatory tissue but not from draining lymph nodes. These findings suggest a more important role of IL-10 in the inflammatory tissue than in secondary lymphoid organs like lymph nodes. More importantly, in the present context, they show the potential utility of the *tiger* system in this kind of study.

In conclusion, we have developed a system to detect and monitor cells committed to IL-10 production. IL-10 production was preferentially observed in some populations of intestinal tissue. Further analysis of how IL-10 expression is obtained in mucosal organs might in turn lead to a better understanding of the suppression

mechanisms used by intestinal lymphocytes and, in the long term, lead to new approaches to the therapy of inflammatory bowel disease as well as vaccine development through the mucosal route. We anticipate that the *tiger* mouse will facilitate the further study of IL-10 biology.

Experimental Procedures

Generation of *tiger* Mice

A 5.8 kb HindIII genomic fragment containing the polyadenylation signal of exon 5 of the IL10 locus was cloned into the HindIII site of the pEasyFlox vector. The BglII-BamH1 fragment and IRES-GFP cassette was isolated from the pMSCV GFP vector and cloned into the BamH1 site of pEasyFlox. With Not1 sites introduced on either end, a 3.8 kb fragment spanning intron 1 through the 3′ untranslated region of exon 5 of the IL10 gene was then inserted into the modified pEasyFlox vector via Not1 sites. The linearized vector was electroporated into TC1 embryonic stem (ES) cells. G418-resistant, gancyclovir-sensitive clones were screened by Southern blot, and selected clones were subsequently transfected with Cre recombinase for neo deletion. Successfully targeted clones were injected into C57BL/6 blastocysts. Chimeric males in which germline transmission was successful were bred to C57BL/6 mice. FIR mice (Wan and Flavell, 2005) were screened by PCR and used to cross with *tiger* mice for the analysis. All mouse experiments were approved by Institutional Animal Care and Use Committee of Yale University.

Th1, Th2 Differentiation In Vitro

$5–15 \times 10^4$ of $CD4^+$ T cells purified with MACS (Miltenyi) and 5×10^6 of APCs (3000 rad irradiated splenocytes) were cocultured with anti-CD3 (145-2C11, ATCC) and anti-CD28 in medium (Bruff's supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin) for times indicated under Th0, Th1 (IL12 plus anti-IL4), or Th2 (IL4 plus anti-IFNγ) conditions (IL12 was a generous gift of Wyeth). Supernatants from these cultures were collected and ELISA was performed.

For intracellular cytokine staining, the cells were washed once and restimulated with PMA (Sigma, 2 ng/ml) and ionomycin (Sigma, 2 μg/ml) for 4 hr. Golgistop (BD, USA) was added during the last 2 hr of restimulation. Intracellular cytokine staining was performed according to the manufacturer's instruction, and the cells were detected for cytokine and GFP on a FACS Calibur instrument (BD, USA). Anti-IL-2, anti-IL-4, anti-IL-10, and anti-IFN-γ for intracellular cytokine staining were purchased from BD, USA. The anti-Foxp3 intracellular staining kit was purchased from eBioscience, USA.

For quantitative PCR, RNA was extracted with TRIzol reagent and cDNA was synthesized by Superscript II reverse-transcriptase according to the manufacturer's protocols (Invitrogen). The ABI 7500 Real-Time PCR system was used for quantitative PCR with primer-probe sets purchased from ABI.

Generation of Bone Marrow-Derived Macrophages and Dendritic Cells

Bone marrow was obtained by flushing tibia and femur with DMEM (Invitrogen). The medium was DMEM supplemented with 20% FCS, glutamine (both from Invitrogen), and, to generate BMDM, 30% L929 supernatant containing macrophage-stimulating factor. Bone marrow cells were cultured for 5 days. Cells were harvested with cold dPBS (Invitrogen), washed, and resuspended in DMEM supplemented with 10% FCS.

To obtain dendritic cells, bone marrow cells were cultured in 3% L929 supernatant containing GM-CSF. Fresh medium was added every 2 days. After 5 days, medium alone or LPS (500 ng/ml), zymosan (50 μg/ml, Sigma), TGF-β (2 ng/ml), and IL-4 (2500 U/ml) were added to the culture, and GFP expression was detected 24 hr later. Dendritic cells from the spleen and Peyer's patch were isolated as reported previously (Iwasaki and Kelsall, 1999).

In Vivo Stimulation of T Cells and Isolation of Lymphocytes

tiger mice and control littermates were injected with 20 μg of anti-CD3 (145 2C11) or nonmitogenic anti-CD3 IgG3 (Alegre et al., 1995) i.p. Alternatively, *tiger* mice crossed to AND TCR Tg mice (Jackson Laboratory, USA) were injected with 5 μg of SEA (Toxin technology, USA) i.p. 1–3 times with an interval of 2 days and sacrificed 4 hr after final injection. For the controls, PBS were injected.

The IEL and LPL were collected as described with some modifications (Das et al., 2003). In brief, small or large intestine was washed with Hank's Buffered Saline and incubated in the presence of 5 mM of EDTA at 37°C for 30 min. The released cells were loaded onto a Percoll gradient and centrifuged. The cells between 44% and 67% Percoll were collected and used as intestinal epithelial cells.

LPL were collected by digesting gut tissue, which was removed for IEL isolation as described above. The tissue was digested with collagenase IV (100 U, Sigma) at 37°C for 1 hr and loaded onto a Percoll gradient and centrifuged. The cells between 40% and 100% Percoll were collected and used as LPL.

Adoptive Transfer of $CD4^+$ T Cells

$CD4^+$ T cells from splenocytes were collected from *tiger* mice and their control wild-type mice by MACS (Miltenyi). 10^6 $CD4^+$ T cells were intravenously injected into *Rag1*$^{-/-}$ mice (Jackson). 1 month later, the mice were injected with 10 μg of anti-CD3 i.p. and the IEL were recovered and examined for GFP by FACS.

Infection with *S. typhimurium* and *L. major*

S. typhimurium infections of streptomycin-treated mice were carried out as previously described (Lara-Tejero et al., 2006). Mice utilized in these studies were confirmed to be *nramp1* (Slc11a1)$^{-/-}$ and therefore of the "*Salmonella*-susceptible" genotype. In brief, mice were pretreated with 20 mg streptomycin 24 hr prior to oral inoculation with either 10^8 or 10^5 c.f.u. of wild-type *S. typhimuium* strain SL1344 and sacrificed 2 and 5 days after inoculation, respectively. The lymphocytes from colon Lamina propria, IEL from small intestine, lymphocytes from spleen, mesenteric lymph nodes, and Peyer's patches were recovered and examined for GFP expression by FACS.

For infection study with *Leishmania*, mice were injected in the hind footpad with 1.5×10^6 stationary-phase *L. major* (WHO MHOM/IL/80/Friedlin) promastigotes and were sacrificed 28 days after infection. Lymphocytes from draining lymph nodes and footpad tissue was recovered and examined for GFP by FACS. To recover lymphocytes from the footpad tissue, tissue was minced and digested with 200 U/ml collagenase IV (Sigma, USA) in DMEM with 10% FCS for 2 hr, and live cells were recovered by step gradient centrifugation with Ficoll (LSM Lymphocyte Separation Medium, MP Biomedicals, OH).

Supplemental Data

Three Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/6/941/DC1/.

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