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A Central Role for Induced Regulatory T Cells in Tolerance Induction in Experimental Colitis[1]

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Abstract

In addition to thymus-derived or natural T regulatory (nT_{reg}) cells, a second subset of induced T regulatory (iT_{reg}) cells arises de novo from conventional $CD4^+$ T cells in the periphery. The function of iT_{reg} cells in tolerance was examined in a $CD45RB^{high}CD4^+$ T cell transfer model of colitis. In situ-generated iT_{reg} cells were similar to nT_{reg} cells in their capacity to suppress T cell proliferation in vitro and their absence in vivo accelerated bowel disease. Treatment with nT_{reg} cells resolved the colitis, but only when iT_{reg} cells were also present. Although iT_{reg} cells required Foxp3 for suppressive activity and phenotypic stability, their gene expression profile was distinct from the established nT_{reg} "genetic signature," indicative of developmental and possibly mechanistic differences. These results identified a functional role for iT_{reg} cells in vivo and demonstrated that both iT_{reg} and nT_{reg} cells can act in concert to maintain tolerance.

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Mature CD4$^+$ "conventional" T (T_{conv})[4] cells isolated from peripheral blood and lymphoid organs can be induced to express Foxp3 in vitro by T cell activation in the presence of TGF-β1 and IL-2 (1–4). This process is enhanced by retinoic acid and antagonized by IL-6 and IFN-γ. Foxp3$^+$ "induced" regulatory T (iT_{reg}) cells have suppressive function, as measured by their capacity to inhibit T cell proliferation in vitro and to inhibit the induction of experimental autoimmune disease. TGF-β1 also acts with IL-6 to induce a T cell-specific isoform of the retinoic acid-related orphan receptor-γ (RORγt), which is necessary for the development of proinflammatory Th17 cells. Foxp3 and RORγt can be expressed in the same cell population and therefore lie at a critical crossroad between inflammation and tolerance.

In TGF-β1 and conditional T cell-specific TGF-β receptor II knockout mice, a lethal lymphoproliferative autoimmune disease, similar to that seen in Foxp3 knockout mice, develops within the neonatal period (5–9). Although "natural" regulatory T (nT_{reg}) cell development in the thymus appears normal, survival in the periphery is compromised (7–9). Furthermore, TGF-β deficiency may abrogate the conversion of T_{conv} cells into iT_{reg} cells. These observations demonstrate an important role for TGF-β in maintaining the regulatory T (T_{reg}) cell pool, but do not functionally discriminate between the two types of T_{reg} cells.

Recent studies have examined the in situ generation of iT_{reg} cells under a variety of different conditions. Chronic exposure to systemically delivered submitogenic amounts of agonistic peptides or T cell expansion in a lymphopenic environment results in Foxp3 expression in up to 15% of Ag-experienced T_{conv} cells. These cells have the capacity to suppress T cell proliferation (10–13). However, studies in diabetic NOD mice with a high frequency of self-reactive T cells showed little overlap in the T_{conv} and nT_{reg} cell TCR pools, suggesting peripheral conversion plays a minimal role in tolerance (14). Peripheral conversion was also not observed after an acute viral infection and only modestly following immunization (15, 16). Arguably, the published data support the interpretation that chronic Ag exposure creates conditions that favor iT_{reg} cell generation and survival, while during primary responses iT_{reg} cells seem to contribute marginally to T_{reg} cell control. Nevertheless, these studies left unresolved the genetic and functional relationships between iT_{reg} and nT_{reg} cells, as well as the biological relevance of the conversion process itself.

In this study, we used mice that express enhanced GFP (EGFP) under the control of the endogenous Foxp3 promoter with either a functional or a nonfunctional Foxp3 protein (*Foxp3*^{EGFP} or *Foxp3*$^{\Delta EGFP}$ mice, respectively) to examine in situ conversion and the contribution of iT_{reg} cells to tolerance. As a test of function, we compared the capacity of iT_{reg} and nT_{reg} cells to modify autoimmune inflammation in a lymphopenia-induced model of inflammatory bowel disease. We also compared the gene expression profiles of iT_{reg} and nT_{reg} cells. We found a synergistic role for iT_{reg} cells in the maintenance of dominant immunological tolerance that depended upon Foxp3 to stabilize differentiation induced by T cell activation and TGF-β1.

[4]Abbreviations used in this paper: T_{conv}, conventional T; T_{reg}, regulatory T; iT_{reg}, induced T_{reg}; nT_{reg}, natural Treg; EGFP, enhanced GFP; RORγt, retinoic acid-related orphan receptor γt.

Materials and Methods

Mice

Foxp3^{EGFP} and *Foxp3*$^{\Delta EGFP}$ mice on the BALB/c background were generated and screened as previously described (16–18). Thy1.1^{+} *Foxp3*$^{\Delta EGFP}$ newborn mice were rescued by i.p. transfer of 60 × 10^{6} unfractionated Thy1.2^{+} BALB/c splenocytes to generate naive Thy1.1^{+}CD4^{+} CD45RB^{high} T cells with the nonfunctional *Foxp3*$^{\Delta EGFP}$ allele. *Rag1*$^{-/-}$ mice were obtained from The Jackson Laboratory. The Animal Resource Committees at the Medical College of Wisconsin and at the University of California, Los Angeles, approved all animal experiments, and moribund mice were sacrificed and analyzed per these protocols.

Cell purification and adoptive transfer

Pooled splenocytes and lymph node cells were stained with either anti-CD4-PE or anti-CD4-Pacific Blue and anti-CD45RB-allophycocyanin as appropriate and sorted on the basis of Ab and EGFP fluorescence. All sorting was done on a FACSAria (BD Biosciences). The average purity and viability of the sorted CD4^{+} populations was 98.8 ± 0.2% and 87.6 ± 1.6%, respectively (SEM, n = 23). Colitis was induced in 6-wk-old BALB/c mice by i.p. injection of 4 × 10^{5} CD4^{+}EGFP^{-}CD45RB^{high} cells suspended in 1 ml of PBS. Following this injection, mice were weighed twice weekly. In some experiments, when mice had lost 5–7% of their initial body weight, they were treated by i.p. injection of T_{reg} cells purified by cell sorting.

Analytical flow cytometry

Cells were stained as described previously (18) and analyzed using an LSRII (BD Biosciences) and FlowJo software (Tree Star).

TGF-β1-mediated in vitro conversion

Sorted EGFP^{-} splenocytes from Foxp3^{EGFP} or Foxp$^{\Delta EGFP}$ mice (1 × 10^{6}/ml) were cultured in anti-CD3 mAb (clone 14-2C11 at 2.5 μg/ml)-coated dishes in the presence of soluble anti-CD28 mAb (clone 37.51) at 1 μg/ml with or without TGF-β1 (5 ng/ml; R&D Systems). After 72 h, cells were either analyzed by flow cytometry or resorted based upon EGFP fluorescence.

Suppression assay

BALB/c splenocytes containing 2.5 × 10^{5} responder CD4^{+} T cells were cultured in 96-well flat-bottom plates in the presence of 1 μg/ml anti-CD3 mAb. The number of responder CD4^{+} cells was kept constant, while the number of suppressor T_{reg} cells was titrated to achieve the indicated ratios in triplicate wells. Cultures were maintained for 48 h, then pulsed with 0.4 μCi/well [^{3}H]TdR for an additional 18 h, harvested, and counted.

Histology

Complete colons were fixed in formalin, processed, and stained with H&E using a histology core facility. Blinded sections from the entire colon were examined by a pathologist (N.H.S.) and large intestine colitis scores were determined for the following inflammatory

changes on a 4-point semiquantitative scale with 0 representing no change (19). The following features were considered: severity, depth and chronic nature of the inflammatory infiltrate, crypt abscess formation, granulomatous inflammation, epithelial cell hyperplasia, mucin depletion, ulceration, and crypt loss.

Statistics

To compare groups with respect to the colitis score, nonparametric methods were used because the data were skewed and ordinal. A Kruskal-Wallis test overall was used with a Mann-Whitney *U* test for pairwise comparison. Since there were ties, the results were checked for consistency using a Savage test or ordered data or a Cochran-Armitage Savage trend test, whichever was relevant. Lymphocyte numbers and lymphocyte percentages were compared by a two-tailed Student's *t* test. To compare percent weight change over time, a random coefficient regression model was used. In the first 10 days, there was little difference between the groups. Analysis was done after day 10. Where the data were nonlinear, a polynomial was fit. The random coefficient model essentially fits each mouse separately and calculates an aggregate weighing with respect to fit of the data to the hypothesized model. Post hoc pairwise comparisons were done using a contrast. No adjustment was made for multiple comparisons. Kaplan-Meier survival curves were compared using the log-rank test.

Gene expression profiling

Purified cells sorted by flow cytometry from 4 to 10 mice were used to generate total RNA, which was amplified and used to create probes for Affymetrix 430 2.0 chips. The subset of probe sets whose expression increased or decreased by 2-fold or more was identified and used for further analysis. The data were normalized using the justRMA algorithm from the Bioconductor group (20). Results represent mean fold change values derived from three to five independent arrays for each cell type and scored $p < 0.05$ by Student's unpaired two-tailed *t* test. Selected targets were verified by flow cytometry. It should be noted that because the gene array probes for *Foxp3* lie distal to the poly(A) start site in the *Foxp3*^{EGFP} allele, a false-negative result is generated for *Foxp3* in *Foxp3*^{EGFP} T cells. The following cell populations were analyzed: freshly isolated and in vitro-activated (anti-CD3 mAb plus IL-2) CD4$^+$EGFP$^+$ (nT$_{reg}$) cells and CD4$^+$EGFP$^-$ (T$_{conv}$) cells from *Foxp3*^{EGFP} mice, iT$_{reg}$ cells from *Foxp3*^{EGFP} and *Foxp3*$^{EGFP/+}$ mice, and ΔiTreg cells from *Foxp3*$^{\Delta EGFP/+}$ mice. The microarray data have been deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE14415.

Results

In situ-generated iT$_{reg}$ cells in experimental colitis

To investigate the relative potency of iT$_{reg}$ cells, the capacity of iT$_{reg}$ cells to suppress autoimmune inflammation was examined in a lymphopenic model of inflammatory bowel disease (21). Colitis was induced in *Rag1*$^{-/-}$ recipients by the i.p. transfer of 4×10^5 CD4$^+$EGFP$^-$ Thy1.2$^+$CD45RB^{high} syngeneic T$_{conv}$ cells isolated by cell sorting from *Foxp3*^{EGFP} or *Foxp3*$^{\Delta EGFP}$ male mice. In the recipients of naive T$_{conv}$ cells from *Foxp3*^{EGFP} mice, diarrhea and weight loss began ~30 days after transfer. Disease progressed until mice

became moribund and were sacrificed ~100 days after transfer (Fig. 1, *A* and *B*). Colitis was marked by extensive leukocytic infiltration of the mucosa, submucosa, muscularis and serosal layers, absence of mucin-secreting goblet cells, epithelial cell hyperplasia with increased crypt length, epithelial cell ulceration, and crypt abscess formation (Fig. 1*C*). The mean colitis score from 12 randomly selected moribund mice was 3.8 ± 0.1 (Fig. 1*D*). Analysis of lymphocytes in the mesenteric lymph nodes 30 days after transfer revealed that 2.4 ± 0.2% of $CD4^+$ T_{conv} cells had become $EGFP^+$, reflecting the in situ generation of iT_{reg} cells ($n = 5$; data not shown). This proportion increased to 9 ± 1.3% of $CD4^+$ T cells at the conclusion of the experiment (Fig. 1*E*). A similar proportion of T_{reg} cells was seen in the mesenteric lymph nodes of normal *Foxp3*^{EGFP} mice, although the total number of $EGFP^+$ cells was reduced in mice with colitis by ~50% (Fig. 1*F*). In situ-generated iT_{reg} cells and nT_{reg} cells from healthy mice had similar suppressive function in vitro, as measured by their capacity to reduce Ab-mediated T cell proliferation. A 50:50 mixture of the two cell types did not increase the suppressive effect over that mediated by iT_{reg} cells alone (Fig. 1*G*). In situ-generated iT_{reg} cells in the mesenteric lymph nodes of mice with colitis could be distinguished from mesenteric lymph node nT_{reg} cells from normal mice on the basis of several cell surface markers including CD25, CD62, and CD103 (Fig. 1*H*).

To determine the extent to which in situ-generated iT_{reg} cells might influence disease progression, we induced colitis with 4×10^5 $EGFP^-Thy1.2^+CD45RB^{high}$ syngeneic T_{conv} cells isolated by cell sorting from *Foxp3*$^{\Delta EGFP}$ mice. For these experiments, *Foxp3*$^{\Delta EGFP}$ mice were rescued at birth with 60×10^6 unfractionated $Thy1.1^+$ splenocytes to allow normal development of the host $Thy1.2^+CD4^+$ T_{conv} cells (our unpublished data). In the absence of a functional *Foxp3* locus, weight loss began ~25 days after transfer and was accelerated relative to recipients of $CD45RB^{high}$ cells from *Foxp3*^{EGFP} mice (Fig. 1*A*, $p < 0.001$). All mice became moribund and were sacrificed 50 days after initiation of the experiment, demonstrating a significant decrease in survival (Fig. 1*B*, $p < 0.001$). Colitis was severe, with a mean colitis score of 3.9 ± 0.1 (Fig. 1, *C* and *D*). Flow cytometry revealed a significant decrease in the percent and number of $EGFP^+$ cells (Fig. 1, *E* and *F*). In situ-derived ΔiT_{reg} cells generated from *Foxp3*$^{\Delta EGFP}$ T_{conv} cells had no suppressive function in vitro (data not shown), although their cell surface phenotype was similar to the in situ-derived iT_{reg} cells generated from *Foxp3*^{EGFP} T_{conv} cells (Fig. 1*H*). Thus, Foxp3 is required to sustain iT_{reg} cell numbers and to develop iT_{reg} suppressive function.

Treating established colitis by T_{reg} cell adoptive transfer immunotherapy

In the preceding experiments, the iT_{reg} cells generated in situ from a starting pool of 4×10^5 $CD45RB^{high}$ T_{conv} cells delayed disease progression and prolonged survival, but were not sufficient to prevent severe colitis. Cotransfer of either iT_{reg} or nT_{reg} cells with the $CD45RB^{high}$ T_{conv} cells can prevent disease from developing (22–24). In a setting that must include in situ-generated iT_{reg} cells (Fig. 1), established disease can also be treated by the adoptive transfer of nT_{reg} cells (25). Together, these observations suggest that iT_{reg} cells contribute to tolerance. In the treatment of established disease, it has not been determined whether iT_{reg} and nT_{reg} cells are interchangeable and function in an additive fashion or whether they act synergistically, perhaps by nonredundant mechanisms. To investigate the respective role of iT_{reg} and nT_{reg} cells in therapy, we treated mice at the point where they

lost 5–7% of their initial body weight (approximately day 30) with 1×10^6 $CD4^+Thy1.1^+$ iT_{reg} cells or 1×10^6 $CD4^+Thy1.1^+$ nT_{reg} cells. $EGFP^+$ iT_{reg} cells were generated in vitro by TCR cross-linking in the presence of TGF-β1 and purified by cell sorting. $EGFP^+$ nT_{reg} cells were obtained from the spleens and lymph nodes of *Foxp3*^{EGFP} mice and also purified by cell sorting. The congenic Thy1.1 marker was used to discriminate between the adoptively transferred T_{reg} cells used to treat the mice and those $Thy1.2^+$ iT_{reg} cells generated in situ during the induction of colitis.

When the number of T_{reg} cells was increased by treating with 1×10^6 $CD4^+$ $Thy1.1^+$ iT_{reg} cells, the mice failed to gain weight but survived to complete the experiment at 125 days (Fig. 2, *A* and *B*). Histology showed moderate hypertrophy of the colonic mucosa, distortion of the crypts, and a patchy inflammatory cell infiltrate in the lamina propria (Fig. 2*C*). Colitis scores averaged 3.0 ± 0.5, consistent with less severe disease than in mice containing only in situ-generated iT_{reg} cells (Figs. 1*D* and 2*D*, respectively). In the mesenteric lymph nodes, 9 ± 1.2% of $CD4^+$ T cells were $Thy1.1^+EGFP^+$ (in vitro-derived iT_{reg} cells) and 3 ± 0.5% were $Thy1.1^-EGFP^+$ (in situ-derived iT_{reg} cells) (Fig. 2*E*). On average, a total of 3.3 ± 0.7×10^6 T_{reg} cells were recovered from the mesenteric lymph nodes and spleen of treated mice (Fig. 2*F*).

When mice were treated with 1×10^6 $CD4^+Thy1.1^+$ nT_{reg} cells,, they exhibited weight gain consistent with clinical recovery, although survival was not significantly different than in mice treated with iT_{reg} cells (Fig. 2, *A* and *B*). Histological examination at 125 days showed nearly normal colons, with an average colitis score of 1.0 ± 0.6 (Fig. 2, *C* and *D*). In the mesenteric lymph nodes, 11 ± 1.1% of $CD4^+$ T cells were $Thy1.1^+EGFP^+$ (nT_{reg} cells) and 3 ± 0.4% were $Thy1.1^-EGFP^+$ (in situ-derived iT_{reg} cells) (Fig. 2*E)*. The total number of T_{reg} cells recovered from the mesenteric lymph nodes was less than half that seen in the iT_{reg} cell-treated mice ($1.3 \pm 0.3 \times 10^6$; Fig. 2*F*). Thus, despite a >50% reduction in T_{reg} cell numbers, the combination of in situ-derived iT_{reg} cells and nT_{reg} cells suppressed bowel inflammation and restored tolerance, whereas mice with a T_{reg} cell compartment containing only iT_{reg} cells had chronic disease. These data suggested more effective disease suppression when the T_{reg} compartment was comprised of both iT_{reg} and nT_{reg} cells.

Functional synergy between nT_{reg} and iT_{reg} cells

To dissect the individual contributions of iT_{reg} and nT_{reg} cells and to examine the possibility of synergy between the two cell types, we induced colitis by the adoptive transfer of $Thy1.1^+$ $CD4^+EGFP^-CD45RB^{high}$ T_{conv} cells isolated from *Foxp3*$^{\Delta EGFP}$ mice. In this experiment, functional iT_{reg} cells are not produced in situ (Fig. 1), making it possible to control the T_{reg} cell dose and track each T_{reg} cell population in subsequently treated mice. When the transfer recipients had lost 5–7% of their initial body weight, they were treated with increasing numbers of nT_{reg} cells or with a combination containing iT_{reg} and nT_{reg} cells.

Mice treated with 0.25×10^6 or 0.5×10^6 $Thy1.2^+$ nT_{reg} cells failed to gain weight and had reduced survival (Fig. 3, *A* and *B*). Mice treated with 1×10^6 $Thy1.2^+$ nT_{reg} cells initially gained weight for a short period of time, but then progressively lost weight. Only one animal lived to complete the experiment at 125 days. Histological analysis and colitis scores were

consistent with severe disease in all groups (Fig. 3, *C* and *D*, average colitis score 3.7 ± 0.2). In mesenteric lymph nodes, the percentage of $EGFP^+$ cells within the $CD4^+$ gate increased with the nT_{reg} cell dose, reaching 13 ± 1.7% in mice that received 1×10^6 $Thy1.2^+$ nT_{reg} cells (Fig. 3*E*). Importantly, 80% of mice that received a total dose of 1×10^6 T_{reg} cells comprised of a 1:1 mix of iT_{reg} and nT_{reg} cells gained weight and recovered completely. They had normal colons by histology (average colitis score, 1.2 ± 0.5). In the mesenteric lymph nodes of these mice, 2 ± 0.1% of the $CD4^+$ T cells were $Thy1.1^+EGFP^+$ iT_{reg} cells and 10 ± 0.6% were $Thy1.2^+EGFP^+$ nT_{reg} cells (Fig. 3). The mesenteric lymph nodes and spleens of animals that survived for >50 days contained a similar number of T_{reg} cells, suggesting that homeostatic regulation of the T_{reg} cell compartment size is not tightly linked to the developmental origin of the T_{reg} cells (Fig. 3*F*). Taken together, the data in Figs. 2 and 3 show that the clinical effect of treating with a mixture of both T_{reg} cell types is greater than treating with either iT_{reg} or nT_{reg} cells alone. These data are consistent with the interpretation that iT_{reg} and nT_{reg} cells can act synergistically to establish and maintain tolerance. It seems likely that in situ-derived iT_{reg} cells and nT_{reg} cells also act in concert in a similar fashion.

Gene expression profiles from nT_{reg} and iT_{reg} cells

The genetic basis for the synergistic function of iT_{reg} and nT_{reg} cells was investigated by comparing the gene expression profile of in vitro-generated iT_{reg} cells with that of nT_{reg} cells isolated from *Foxp3*^{EGFP} mice. Results were also compared with other published gene expression profiles of T_{reg} cells and T_{conv} cells (15, 17, 26, 27). We first identified genes common to nT_{reg} and iT_{reg} cells as compared with T_{conv} cells. A total of 155 unique genes were found common to both nT_{reg} and iT_{reg} cells, of which 90 were overexpressed and 65 were underexpressed (Fig. 4*A* and supplemental Table I[5]). Comparing iT_{reg} and in vitro-activated nT_{reg} cells with activated T cells filtered out the contribution of T cell activation, revealing a smaller subset of common genes that included the prototypical T_{reg} cell genes *Socs2*, *Gpr83*, and *Nrp1* (neuropilin) (supplemental Table II). Together, these findings confirmed the overlap of the genetic signatures of nT_{reg} and iT_{reg} cells.

Despite sharing a portion of the T_{reg} genetic signature, nT_{reg} and iT_{reg} cells were genetically distinct, with each subset marked by a large number of differentially expressed genes (Fig. 4, *B* and *C*). A subset of canonical T_{reg} transcripts was selectively expressed in freshly isolated nT_{reg} cells but not in iT_{reg} cells, including the Ikaros family member Helios (*Zfpn1a2*), the calcium-binding protein *S100a4* and *S100a6*, *Klrg1*, *Itgb8*, *Swap70*, *Pim1*, *Ecm1*, and *Sell*. In turn, a large number of transcripts were differentially enriched in iT_{reg} cells, including the serine protease inhibitors *Ctla2a* and *Ctla2b*, *Tnfrsf5* (CD40), *Acvr1* (activin receptor A type I), *Lta* (lymphotoxin α), *Ahr* (aryl hydrocarbon receptor), and *Zbtb32* (repressor of gata) (supplemental Table III). The aryl hydrocarbon receptor is of particular interest given the recent report that activation of the aryl hydrocarbon receptor can generate iT_{reg} cells by a TGF-β1-dependent mechanism and that these iT_{reg} cells protect mice from experimental allergic encephalomyelitis (28). Although a subset of the genes selectively enriched in the freshly isolated nT_{reg} cells was down-regulated upon in vitro cell

[5]The online version of this article contains supplemental material.

activation, the differential expression of several others, including *Zfpn1a2*, *Sell*, and *Swap70* was retained (supplemental Table IV). Comparison with in vitro-activated nT_{reg} cells also revealed the iT_{reg} cells to be enriched in components of TGF-*β*-related pathways, including *Acvr1*, *Tgfbr1*, *Tgfbr3*, *Smurf1*, *Id1*, and *Id2*. Thus, at least some of differences in the gene expression profiles of nT_{reg} and iT_{reg} cells reflected a more fundamental divergence in their genetic programs that could not be attributed to their activation status.

To determine the role of Foxp3 in the induction and maintenance of the iT_{reg} cell genotype, we compared the gene expression profile of successfully derived iT_{reg} cells that expressed high levels of Foxp3 and exhibited potent in vitro suppressive activity with that of $CD4^+$ T cells from the same culture that failed to express Foxp3 (supplemental Table V). We also compared the expression profiles of iT_{reg} cells with ΔiT_{reg} cells derived in vitro from *Foxp3*$^{\Delta EGFP/+}$ mice (Fig. 4*D* and supplemental Table VI). Surprisingly, both comparisons revealed that the genetic signatures of the respective populations were virtually identical. However, when sorted ΔiT_{reg} cells were maintained in culture for 7 days in the presence of IL-2, the expression of a number of genes associated with the T_{reg} signature such as *Gpr83*, *Nrp1*, and *Itgae* was down-regulated (Fig. 4*D* and supplemental Table VII). These findings indicated that, within the set parameters of the analysis, Foxp3 contributed to the subsequent stability but not the initial establishment of the iT_{reg} cell genetic signature, which seems to be primarily shaped by the actions of TGF-*β*1 and TCR signaling (29).

Discussion

The central finding of this study is that iT_{reg} cells form a subdivision within the peripheral T_{reg} cell pool that can act synergistically with nT_{reg} cells to maintain tolerance. Although Foxp3 is required for iT_{reg} cell function and stability, the genetic signature of iT_{reg} cells only partially overlapped that of nT_{reg} cells, consistent with their different ontogeny and possibly nonredundant functional role. The concordant gene expression profiles seen at early time points in iT_{reg} and ΔiT_{reg} cells indicated that Foxp3 played little role in initiating iT_{reg} conversion. However, similar to nT_{reg} cells, the suppressive function of iT_{reg} cells is absolutely dependent upon Foxp3 (17, 30). The eventual loss of EGFP fluorescence in ΔiT_{reg} cells also indicated that stability of the iT_{reg} phenotype requires continued expression of a functional Foxp3 protein. This would explain previous observations that T_{conv} cells from Foxp3$^{\Delta EGFP}$ mice that were transferred into SCID recipients failed to give rise to iT_{reg} cells in vivo, even while inducing an aggressive colitis (17). A similar role has been suggested for Foxp3 in nT_{reg} cell phenotype stability, supporting the idea that a principle function of Foxp3 is to differentially regulate a subset of genes activated by TCR-, IL-2-, and TGF-*β*1-mediated signaling and to sustain its own expression (30).

The apparent dual requirement for iT_{reg} and nT_{reg} cells to enforce peripheral tolerance may reflect the distinct ontogeny and Ag specificity of the respective T_{reg} population. Early in life, iT_{reg} cells may be particularly vital due to the delay in nT_{reg} development relative to the production of T_{conv} cells by the newborn mouse thymus (31). Indeed, early thymectomy removes a large component of the developing nT_{reg} repertoire but leaves intact the capacity of peripheralized T_{conv} cells to express Foxp3, resulting in a milder disease phenotype than seen in Foxp3 deficiency (32). Other evidence theoretically supports the requirement for

iT_{reg} cells. T_{conv} cells, from which iT_{reg} cells are derived, are by-and-large specific for foreign Ags. Conversely, several studies report that the nT_{reg} cell TCR repertoire may be enriched with self-specific receptors (33, 34), with some overlap with that of T_{conv} cells (33–36). Our data suggest that when comparing the nT_{reg} and T_{conv} TCR repertoires, overlap may be largely due to iT_{reg} cells. Expanding clones that produce both T_{conv} and iT_{reg} cell effectors would be predicted to generate the most frequently shared TCR isolates. Even if the molecular mechanisms for iT_{reg}- and nT_{reg} cell-mediated suppression are functionally equivalent, differences in their TCR repertoires could control the type and extent of T_{reg} cell activation. Additionally, local factors including the type of APC and the availability of cofactors like retinoic acid are likely to influence the size and Ag specificity of the iT_{reg} compartment (37–39). The composition of the antigenic environment may therefore be a primary determinant of the proportional contribution of iT_{reg} and nT_{reg} cells to the maintenance of tolerance.

Differences in the genetic signatures of iT_{reg} and nT_{reg} cells provide clues to their respective attributes. For example, Helios, a member of the Ikaros family of zinc finger transcription factors, was enriched in nT_{reg} relative to iT_{reg} cells. Helios is primarily expressed in T-lineage cells and early multipotential precursors (40, 41). It associates with the nucleosome remodeling deacetylase complex, which has been implicated in the activation of CD4 gene expression in developing thymocytes, suggesting a role for Helios-nucleosome remodeling deacetylase complexes in nT_{reg} development and perhaps phenotype stability (42, 43). In contrast, iT_{reg} cells were enriched in components of TGF-β-related pathways, such as the aryl hydrocarbon receptor, which may promote proliferation in the face of intense TGF-β signaling (44), and lymphotoxin-α, whose role in T_{reg} cell function remains unexplored. Both iT_{reg} and nT_{reg} cells differentially expressed a number of homing receptors such as *Sell*, suggesting distinct tissue-homing characteristics. These differences may individually or in combination provide differential effector mechanisms, which, together with their distinct TCR repertoires, enable the two populations to synergistically interact to promote in vivo suppression.

Recent reports demonstrate that nT_{reg} cells have epigenetic changes that are consistent with a stable, differentiated cell lineage (45, 46). Unlike nT_{reg} cells, the iT_{reg} cell compartment is not fixed and is maintained in dynamic equilibrium with T_{conv} cells. For example, in the colitis experiments, functional iT_{reg} cells are clearly generated in situ, while ~60% of the surviving in vitro-derived iT_{reg} cells lost Foxp3 expression. Our experiments are therefore most consistent with a model of T_{reg} cell function that involves a terminally differentiated nT_{reg} cell population augmented by iT_{reg} cells, with the latter entering and leaving the peripheral pool proportional to the needs of the host to control autoinflammatory responses.

Finally, the observation that both iT_{reg} and nT_{reg} cells are necessary to treat active colitis suggests that they may also have non-redundant roles in maintaining tolerance once established. In that regard, nT_{reg} or iT_{reg} depletion experiments in the absence of in situ-generated iT_{reg} cells may help to functionally discriminate between the two cell types. It will also be important to determine whether iT_{reg} cells function at sites other than the mucosal interface, where the microenvironment is conducive to their generation. These investigations will bear on the potential therapeutic use of T_{reg} cells in treating autoimmune diseases and

graft-versus-host disease after bone marrow transplantation (47). Specifically, the use of iT_{reg} cells derived in vitro from T_{conv} cells may not prove sufficient to enforce tolerance, particularly in those situations where the indigenous nT_{reg} cells are either depleted or rendered ineffective. Strategies that promote both nT_{reg} and iT_{reg} responses may have a better chance of success.

Supplementary Material

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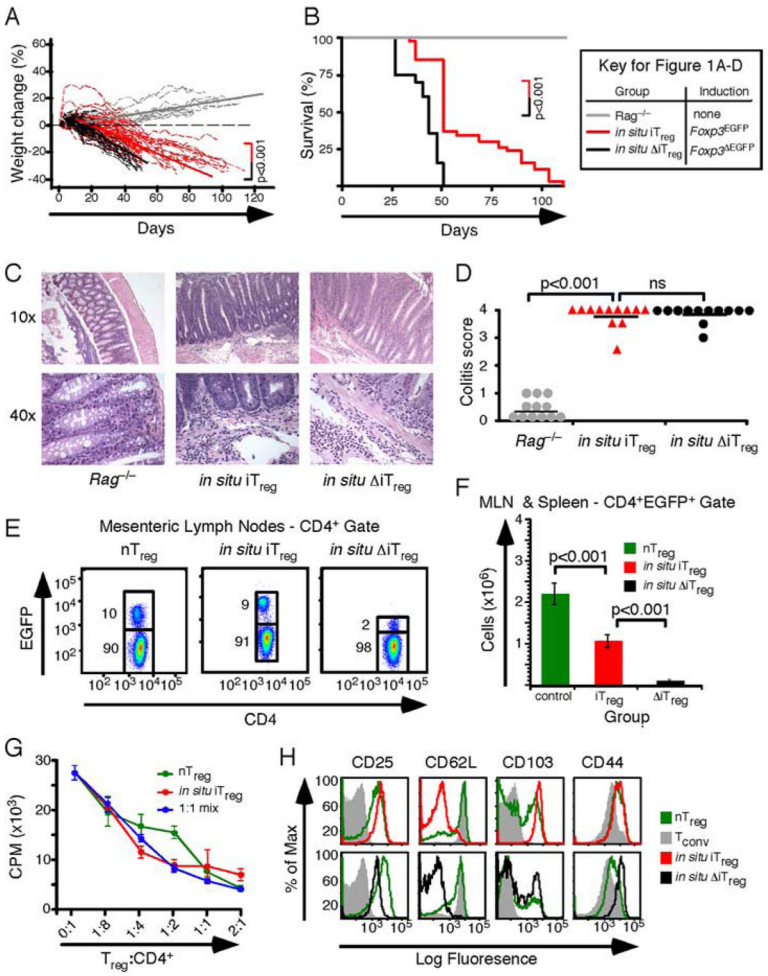

FIGURE 1.

Induction of colitis in the absence of functional Foxp3. *A*, Weight change following the adoptive transfer of 4×10^5 $CD4^+EGFP^-Thy1.2^+CD45RB^{high}$ T cells isolated from *Foxp3*^{EGFP} (dashed red lines; $n = 47$) and Foxp3$^{\Delta EGFP}$ (dashed black lines; $n = 20$) mice into $Rag^{-/-}$ recipients. Control $Rag^{-/-}$ littermates (gray lines; $n = 11$) did not receive transferred cells. Color-matched linear regression lines are plotted for each data set. *B*, Kaplan-Meier survival curves for the mice in *A*. *C*, Representative histological sections from the colons of randomly selected mice in *A* stained with H&E. *D*, Scatter plot showing the colitis score for each mouse where histology was obtained. *E*, Representative flow cytometry analysis of the mesenteric lymph nodes from each group ($n = 7$, 14, and 17, *left* to *right*). Numbers denote means for the adjacent gate. *F*, The total number of T_{reg} cells found in the mesenteric lymph nodes of healthy *Foxp3*^{EGFP} mice (green) and in mice with colitis with either functional (red) or nonfunctional (black) in situ-derived iT_{reg} cells. *G*, Representative data from in vitro suppression assays ($n = 3$) using sorted in situ-derived iT_{reg} cells (red), sorted nT_{reg} cells from healthy mice (green), or a 50:50 mix of each population (blue) to inhibit the anti-TCR-induced proliferation of unfractionated splenocytes. The ratio of T_{reg} cells to $CD4^+$ responder splenocytes is indicated. *H*,

Representative cell surface marker analysis of $CD4^+EGFP^+$ mesenteric lymph node T_{reg} cells from *E* above stained as indicated.

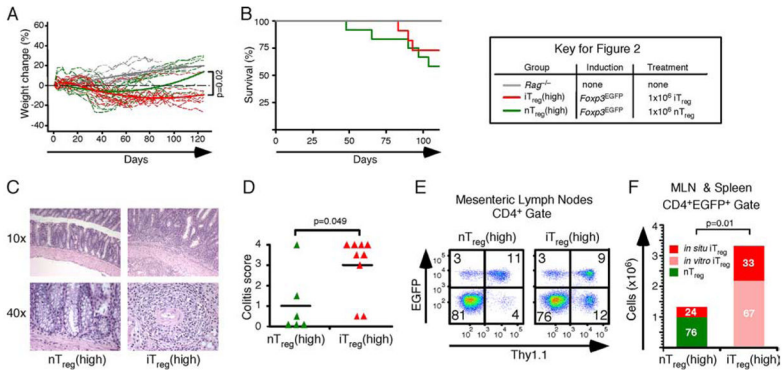

FIGURE 2.

Treatment of colitis in the presence of in situ-derived iT_{reg} cells. *A*, Weight change following colitis induced by adoptive transfer of 4×10^5 $CD4^+EGFP^-Thy1.2^+CD45RB^{high}$ T cells isolated from *Foxp3*^{EGFP} mice. After losing 5–7% of their body weight, recipients were treated by i.p. injection of 1×10^6 sorted in vitro-derived $Thy1.1^+EGFP^+$ iT_{reg} cells (red; $n = 9$) or by 1×10^6 sorted $Thy1.1^+EGFP^+$ nT_{reg} cells (green; $n = 6$). Color-matched solid lines represent the polynomial fit based on a random coefficient regression model. *B*, Kaplan-Meier survival curves for the mice in *A*. *C*, Representative histological sections from the colons of the mice in *A* stained with H&E. *D*, Scatter plots showing colitis scores for each group. *E*, Representative flow cytometry analysis of the mesenteric lymph nodes from mice treated with nT_{reg} cells (*left panel*) or iT_{reg} cells (*right panel*). Numbers denote mean values for the quadrant. *F*, Bar graphs depicting the total number of T_{reg} cells found in the mesenteric lymph nodes and spleens of treated mice. The proportion of in situ-derived iT_{reg} cells (red), nT_{reg} cells (green), and in vitro-derived iT_{reg} cells (pink) is indicated.

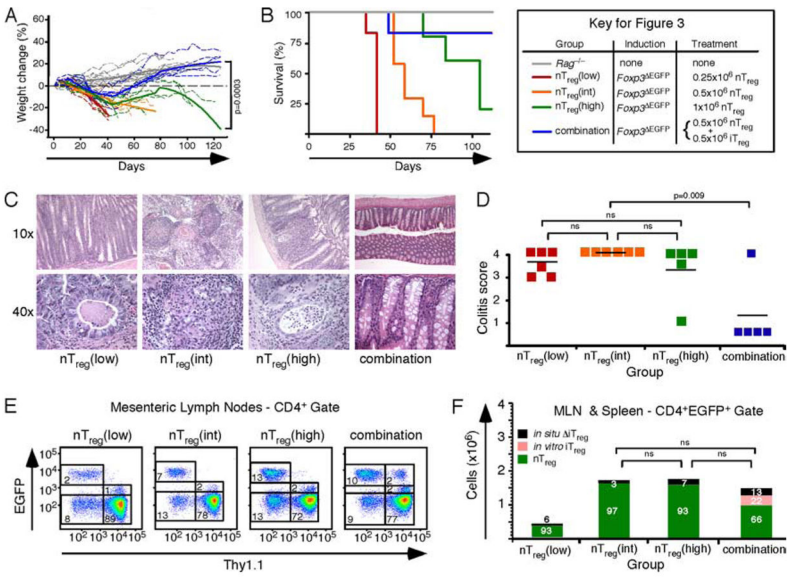

FIGURE 3.

Treatment of colitis in the absence of in situ-derived iT_{reg} cells. *A*, Weight change following colitis induced by adoptive transfer of 4×10^5 $CD4^+EGFP^-$ $Thy1.1^+CD45RB^{high}$ T cells isolated from *Foxp3*$^{\Delta EGFP}$ mice. After 5–7% weight loss, recipients were treated by i.p. injection of 0.25×10^6 $Thy1.2^+EGFP^+$ nT_{reg} cells (red; $n = 6$), by 0.5×10^6 $Thy1.2^+EGFP^+$ nT_{reg} cells (orange; $n = 6$), by 1×10^6 $Thy1.2^+EGFP^+$ nT_{reg} cells (green; $n = 5$), or by a mixture of 0.5×10^6 $Thy1.1^+$ iT_{reg} and 0.5×10^6 $Thy1.2^+nT_{reg}$ cells (blue; $n = 5$). Color-matched solid lines represent the polynomial fit based on a random coefficient regression model. *B*, Kaplan-Meier survival curves for the mice in *A*. *C*, Representative histological sections from the colons of the mice in *A* stained with H&E. *D*, Scatter plots depicting the colitis scores for each group. *E*, Representative flow cytometry analysis of the mesenteric lymph nodes from mice treated with nT_{reg} cells (*left panels*) or a mixture of both cell types (*right panel*). Numbers denote mean values for the quadrant or gate. *F*, Bar graphs depicting the total number of T_{reg} cells found in the mesenteric lymph nodes and spleens of treated mice. The proportion of nonfunctional in situ-derived ΔiT_{reg} cells (black), nT_{reg} cells (green), and in vitro-derived iT_{reg} cells (pink) is indicated. ns, Not significant).

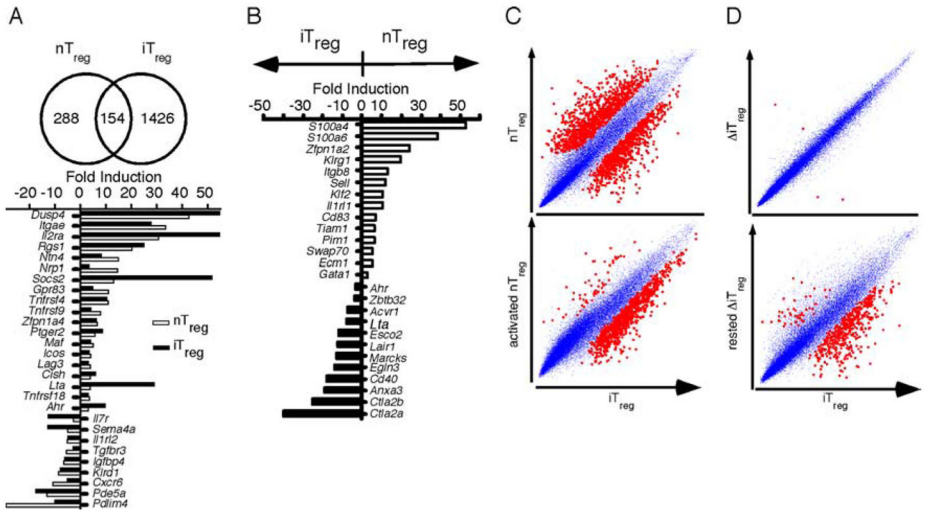

FIGURE 4.

Overlap among the gene expression profiles of iT_{reg} cells and nT_{reg} cells. A comparison of the expression profiles of iT_{reg} and nT_{reg} cells with that of T_{conv} cells identified commonly (*A*) and selectively (*B*) expressed genes. All cells were derived from *Foxp3*^{EGFP} mice. The Venn diagrams illustrate the comparison groups and the number of distinct and shared genes. The bar graphs show a few of the genes that are differentially expressed in iT_{reg} and nT_{reg} cells as mean fold change values derived from three to five independent experiments, each representing mRNA pooled from 4 to 10 mice and scoring $p < 0.05$ by Student's unpaired two-tailed *t* test. *C*, Scatter plots of mean gene expression values of iT_{reg} cells vs nT_{reg} cells (*top panel*) or activated nT_{reg} cells (*bottom panel*). *D*, Scatter plots of mean gene expression values of iT_{reg} cells vs ΔiT_{reg} cells (*top panel*) or iT_{reg} cells vs ΔiT_{reg} cells cultured for 7 days in 50 IU/ml IL-2 (*bottom panel*). Gene comparisons that are found significant in *C* and *D* are highlighted in red.