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Cross-differentiation from the CD8 lineage to CD4 T cells in the gut-associated microenvironment with a nonessential role of microbiota

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SUMMARY

CD4 and CD8 T-cell lineages differentiate through respective thymic selection processes. Here we report cross-differentiation from the CD8 lineage to CD4 T cells, but not vice versa, predominantly in the large-intestine-associated microenvironment. It occurred in the absence or distal presence of cognate antigens. This pathway produced MHC-class-I-restricted $CD4^+Foxp3^+$ T_{reg} ($CI\text{-}T_{reg}$) cells. Blocking T-cell-intrinsic TGFβ signaling diminished $CI\text{-}T_{reg}$ populations in lamina propria but did not preclude the CD8-to-CD4 conversion. Microbiota were not required for the cross-differentiation but presence of microbiota led to expansion of the converted CD4 T-cell population in the large intestine. $CI\text{-}T_{reg}$ cells did not promote tolerance to microbiota per se, but regulated systemic homeostasis of T lymphocytes and protected the large intestine from inflammatory damage. Overall, the clonal conversion from the CD8 lineage to CD4 T-cell subsets occurred regardless of “self” or “nonself”. This lineage plasticity may promote “selfless” tolerance for immune balance.

INTRODUCTION

The development of the immune system has been largely characterized on the basis of discriminating “self”, the host’s own cells, from “nonself”, exemplified by infectious microbes, towards an outcome of either tolerance or immunity (Burnet, 1957). The gut-associated environment (**GAE**), particularly the large intestine, presents a unique challenge to the immune system with a diversity of food antigens and a superior number of normal floral microorganisms (microbiota) that carry a microbial pattern otherwise typified for initiating immunity (Janeway and Medzhitov, 2002). Directly or indirectly, microbiota

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SUPPLEMENTAL INFORMATION

Supplemental information includes 7 figures and Supplemental Procedures.

DISCLOSURE

The authors declare no competing financial interest.

affects development of gastrointestinal tract and the host immune system, and performs a number of functions that are beneficial to the host (Hooper and Macpherson, 2010). Thus, a harmonious relationship between the immune system, microbiota and food antigens in the large-intestine-associated microenvironment is vital for the health of a mammalian host.

In vertebrates, the innate immune system discriminates microbial agents by patterns that are distinct from eukaryotic cells, whereas the adaptive immune system is armed with a repertoire of T and B lymphocyte clones with fine specificity to foreign antigens but is tolerant toward the host's "self" tissue. The "self"-based concept has served as a foundation for modern immunology but its limitations have long been recognized (Matzinger, 1994). How the immune system deals with mutualistic and massive microbiota in the large intestine remains a problem of extensive interest. Extrathymic $CD4^+Foxp3^+$ regulatory T (T_{reg}) cells that developed in the periphery through TGFβ signaling were shown to have a critical role in maintaining tolerance at the mucosal surface including in the large intestine (Josefowicz et al., 2012). Indeed, T_{reg} cell clones specific to microbial agents in the large intestine were identified, and the unique repertoire of colonic T_{reg} cells suggested that the differentiation of peripheral T_{reg} cells could occur locally at the intestinal mucosal surface (Lathrop et al., 2011). However, sequencing analyses of the T-cell antigen receptor (**TCR**) of colonic T_{reg} cells using the TCR^{mini} model, which was constructed to host a diverse but limited repertoire to enable the sequencing studies, suggested that thymus-derived T_{reg} cells may be mainly responsible for tolerance induction to the large intestine microbiota (Cebula et al., 2013).

Nevertheless, one might argue that specific-antigen-based tolerance to microbial organisms must be limited in scope, because a constitutive tolerance toward a broad spectrum of nonpathogenic bacteria can potentially cripple immunity against pathogenic bacteria, which differ minimally from the former in terms of patterns for immune initiation. Indeed, continued presence of microbiota may promote protective immunity overall, as demonstrated in a recent study showing that antibiotic depletion of microbiota impaired antiviral innate and adaptive immunity (Abt et al., 2012).

Therefore, although the CD4 and CD8 lineage specification of αβ T cells occurs in the thymus as a result of a multi-stage, stringent selection process involving recognition of class-I or -II MHC molecules (Doyle and Strominger, 1987; Hedrick, 2012; Norment et al., 1988; Rudd et al., 1988; Veillette et al., 1988), it is possible that in the large-intestine-associated microenvironment, evolution might have shaped unique mechanisms of T-cell plasticity that might not be constrained by "self" versus "nonself" characterization of specific-antigen recognition. We hypothesize that T-cell clones in the large-intestine-associated microenvironment can differentiate at steady-state with lineage plasticity to facilitate immune balance, without regard to "self" or "nonself" denotation of their TCR specificity.

To test this hypothesis, we examined the steady-state T-cell differentiation in the large-intestine-associated microenvironment, tracking the fate of two clones in the CD8 T-cell lineage and two clones in the CD4 T-cell lineage, specific to neither microbiota nor food antigens. Their known specific antigens were either absent in the whole animal or present as

a “self” antigen in an organ (the pancreas) distal to GAE. We used the OTI TCR-transgenic model (Hogquist et al., 1994) with *Rag1* knockout (Mombaerts et al., 1992) ($OT1^+Rag^o$), which renders a T-cell repertoire consisting of a single clone of the MHC-class-I restricted CD8 lineage specific to chicken ovalbumin (absent in the mice or their diet). We also used the 8.3 TCR-transgenic model (Verdaguer et al., 1997) on Rag1-deficient (Mombaerts et al., 1992) background (8.3^+Rag^o), which hosts a monoclonal repertoire of the CD8 lineage specific to a “self” antigen that is not in GAE but in the endocrine pancreas. CD4 lineage plasticity in the large-intestine-associated microenvironment was examined with monoclonal TCR-transgenic models in settings analogous to that established for tracking the clonal fate of the CD8 lineage. Although the monoclonal TCR-transgenic model was necessary to track T-cell lineage fate at clonal level *in vivo* in terms of antigen-specificity, or lack thereof, the exaggerated simplicity of repertoires in this reductionist approach raised question about its physiological relevance. Therefore, the plasticity of the CD8 T-cell lineage was corroborated using T cells with a natural repertoire, in adoptive transfer settings as well as under a condition of immune imbalance simulated by CD4 depletion.

RESULTS

Cross differentiation without regard to selfness

$OT1^+Rag^o$ mice on the C57BL/6 (B6) genetic background, a widely used model in immunological studies, are thought to harbor a monoclone of CD8 T cells with TCR restricted to MHC-class-I recognition. We analyzed adult $OT1^+Rag^o$ B6 mice for T-cell differentiation in GAE. Surprisingly, we found a large population of CD4 T cells (~10–20%) in the large intestine lamina propria (LILP), with a vast majority expressing Foxp3, the transcription factor specifying the $CD4^+$ T_{reg} cell lineage (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells also existed as substantial populations in the mesenteric lymph node (MLN) of $OT1^+Rag^o$ mice, but were infrequent in the other organs (Figure 1A). We have analyzed five $OT1^+Rag^o$ mice from 3- to 7 wks of age and did not detect CD4 T-cell populations in intra-epithelial lymphocyte (IEL) preparations of the large or small intestines (not shown). The small intestine lamina propria (SILP) (not shown) in three of the five $OT1^+Rag^o$ mice had CD4 T cells but the percentage of the CD4 T-cell population was 2–10-fold less than that in LILP.

We next examined the clonal fate of 8.3^+Rag^o T cells in the large-intestine-associated microenvironment. We detected low frequencies of $CD4^+$, but not $CD4^+Foxp3^+$, T cells in 8.3^+Rag^o mice on the NOD genetic background (Figure S1A–B). However, in 8.3^+Rag^o mice on the NOD/B6 mixed genetic background (NOD/B6.8.3^+Rag^o), we found substantial populations of $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells. Interestingly, $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells were detected in the LILP and MLN but not in the pancreatic lymph node (PLN), despite the presence of natural self-antigens specific for the 8.3 clone in the pancreas (Verdaguer et al., 1997) (Figure 1B; Figure S1A–B). Homozygosity of the NOD MHC locus on the NOD/B6 mixed genetic background did not preclude the cross-differentiation, and thus was unlikely responsible for the lack of the cross-differentiated CI-T_{reg} cells in the NOD genetic background (Figure S1C), indicating a non-*MHC* genetic element(s) that influences CD8-to-CD4 cross-differentiation. These observations, together

with the findings in the OT1^{+}Rag^{o} model, suggest that cross-differentiation from the CD8 T-cell lineage to CD4^{+}Foxp3^{-} and CD4^{+}Foxp3^{+} T cells occurs in the large-intestine-associated microenvironment regardless of the lack of, or distance to, cognate antigens.

Conversion from the CD8 lineage to CD4 T cells with MHC-class-I-restricted TCR clonotype

The OT1 clonotypic TCR is restricted to MHC-class-I recognition. Using Rag-deficient OT1 transgenic mice, previous studies established that MHC-class-I-based positive selection is essential for maturation of OT1 T-cell clone to the CD8 lineage in the thymus (Kirchner and Bevan, 1999). To examine the TCR clonotype of the CD4^{+} and CD4^{+}Foxp3^{+} cells in the OT1^{+}Rag^{o} mice, we did flow cytometry analyses of the TCR α and β chain. Indeed, CD4^{+} and CD4^{+}Foxp3^{+} cells in the OT1^{+}Rag^{o} mice expressed the OT1 clonotypic TCR (Figure S2A). In addition, the CD4 T cells produced in OT1^{+}Rag^{o} mice responded to stimulation with the MHC-class-I-restricted peptide (SIINFEKL) of the chicken ovalbumin and proliferated like the CD8 OT1 cells (Figure S2B). *In vivo*, purified CD4 OT1 cells adoptively transferred into B6 hosts responded to immunization with SIINFEKL peptide (Figure S2C). Therefore, the CD4^{+}Foxp3^{-} and CD4^{+}Foxp3^{+} subsets cross-differentiated from the CD8 lineage differed from the MHC-class-II-restricted CD4 T-cell subsets associated with the gut environment in previous studies (Esplugues et al., 2011; Ivanov et al., 2009; Wu et al., 2010), in their MHC-class-I restricted responses. The development of MHC-class-I-restricted CD4^{+}Foxp3^{+} T_{reg} (**CI-T_{reg}**) cells represents a novel pathway of CD4^{+} T_{reg} cell generation in the periphery.

With regard to their differentiation of effector function, the cross-differentiated CD4 T cells is also distinct from typical gut-associated, MHC-Class-II-restricted CD4 T cells (Esplugues et al., 2011; Ivanov et al., 2009; Wu et al., 2010): they were predominantly Th1-like, rather than Th17-like (Figure S2D–E). We also analyzed suppressive cytokine expression in purified OT1 CD4^{+}Foxp3^{+} cells and OT1 CD4^{+}Foxp3^{-} cells, in comparison to that in CD4^{+}Foxp3^{+} cells from B6 mice. By quantitative RT-PCR, we did not detect IL10 mRNA in OT1 CD4^{+}Foxp3^{+} cells, whereas IL10 mRNA levels in OT1 CD4^{+}Foxp3^{-} cells were comparable to that in B6 T_{reg} cells. TGFβ mRNA levels were similar in OT1 CD4^{+}Foxp3^{+}, OT1 CD4^{+}Foxp3^{-} and B6 T_{reg} cells (1.44±0.02, 1.38±0.02 and 1.78±0.5 relative units, respectively).

Lack of lineage plasticity in the MHC-class-II-restricted CD4 T cell clones in the large intestine microenvironment at steady state

To examine whether T-cell clones in the MHC-class-II-restricted CD4 T-cell lineage also have steady-state plasticity in the large-intestine-associated microenvironment, we used models analogous to OT1^{+}Rag^{o} and 8.3^{+}Rag^{o} mice, respectively: OTII TCR-transgenic model (Barnden et al., 1998) deficient in Rag1 (OTII^{+}Rag^{o}), with a monoclonal repertoire of MHC-class-II restricted CD4 T-cell lineage specific to the chicken ovalbumin; BDC2.5 TCR-transgenic model (Katz et al., 1993) deficient in Rag1 (BDC2.5^{+}Rag^{o}), with a monoclonal repertoire of MHC-class-II-restricted CD4 lineage reactive to a natural self-antigen in the endocrine pancreas. We did not detect conversion of the CD4 T-cell clones to CD8^{+} or Foxp3^{+} cells in LILP or MLN (Figure S3).

Two recent studies demonstrated the reprogramming of CD4 T cells to CD8 T cells in the small intestine, triggered by microbial antigens (Mucida et al., 2013; Reis et al., 2013). Those studies suggest novel molecular pathways operating in the small-intestine-associated microenvironment for the generation of $CD4^+CD8\alpha^+$ cytotoxic T lymphocytes (CTLs) restricted to MHC-class-II recognition (Anderson, 2013). Although our studies of T-cell clonal fate with known antigen-specificities were limited to the two clones in the CD4 T-cell lineage, the results suggest that the CD4 T-cell clones may lack lineage plasticity in steady-state of the large-intestine-associated microenvironment, unlike their CD8 counterparts, the OT1 and 8.3 clones, in the same setting. On the other hand, our observations of CI-T_{reg}-cell development in the large-intestine-associated microenvironment provide a parallel to the findings of MHC-class-II-restricted generation of CTLs in the small-intestine-associated microenvironment (Mucida et al., 2013; Reis et al., 2013).

MHC-class-II-dependency of cross-differentiation from the CD8 lineage to CD4 T cells

While the cross-differentiation generated MHC-class-I-restricted CD4 T cells, the cross-differentiation process may require CD4 interaction with MHC-class II (MHCII) molecules. To examine this possibility, we used two independent models (Grusby et al., 1991; Madsen et al., 1999) of MHCII-deficiencies. Indeed, MHCII-deficiencies caused by either H-2Ab knockout or complete knockout of the whole MHCII locus (Grusby et al., 1991; Madsen et al., 1999) abrogated the CD8-to-CD4 cross-differentiation (Figure 2). Possibly, during the cross-differentiation, CD8 silencing and CD4 induction may lead to engagement of both class I and class II of MHC on the same antigen-presenting cell, by the MHC-class-I-restricted TCR and CD4, respectively.

Essential role of MLN but not the thymus nor the immunocompromised status

To identify the organs wherein the cross-differentiation first occurred, we analyzed cohorts of $OT1^+Rag^o$ mice starting when they were young. We did not detect $CD4^+Foxp3^+$ populations in the thymus. $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells first appeared in the MLN and then in LILP around two weeks of age (Figure S4A). This predominant occurrence in the large-intestine-associated microenvironment was also similar to what was found with the $NOD/B6.8.3^+Rag^o$ model (Figure 1B).

The kinetic evidence from both $OT1^+Rag^o$ and 8.3^+Rag^o models suggests a role for MLN in cross-differentiation from the CD8 lineage to CD4 T cells. Previous studies showed that in the MLN, both dendritic cells and stromal cells presented antigens to stimulate OT1 cell responses (Lee et al., 2007). We assessed the role of MLN at a whole organ level by surgical experiments. We surgically removed the MLN in 2-wk-old $OT1^+Rag^o$ mice and analyzed the animals 2 wks later. Removing MLN diminished the $CD4^+Foxp3^+$ population. However, it did not reduce total CD4 T cell counts in $OT1^+Rag^o$ mice (Figure 3A–C).

To determine whether mature CD8 T cells can indeed convert to CD4 T cells without a thymic input, we purified $CD8^+CD4^-CD25^-Foxp3^-$ T cells from the peripheral lymphoid organs (the spleen and lymph nodes) of adult $OT1^+Rag^o$ mice and injected the cells into Foxn1-deficient “nude” mice that lack a functional thymus (Nehls et al., 1994). In the athymic recipients, purified $CD8^+CD4^-CD25^-Foxp3^-$ OT1 T cells efficiently converted to

CD4 T cells, such that in LILP, CD4 T cells, rather than CD8 T cells, predominated (>50%-80%) (Figure 3D). Still, there was a possibility that the purified peripheral CD8 T-cell population contained a fraction of cells “mis-selected” or “imprinted” by MHCII in thymic selection but exported to the peripheral CD8 T-cell repertoire in a metastable state. To test this possibility, we purified CD8 T cells from $OT1^+Rag^oMHCII^o$ or $OT1^+Rag^oMHCII^+$ donors and transferred them into nude mice. As shown in Figure 3D, CD8 T cells from MHC^+ or $MHCII^o$ donors both converted to CD4 T cells efficiently. In this setting of $MHCII^o$, the donor CD8 T cells had never been exposed to MHCII prior to transfer. The recipient mice were athymic. Therefore, the CD8-to-CD4 cross-differentiation reflects peripheral conversion rather than an undetected effect of “mis-selection” or “imprinting” by thymic MHCII.

Conversely, to test whether prior exposure to MHCII potentiates CD8-to-CD4 conversion in a new $MHCII^o$ host, we compared conversion of CD8 T cells from $OT1^+Rag^oMHC^+$ donors in Rag^oMHCII^o versus Rag^oMHCII^+ hosts. The absence of MHCII in the new host abrogated CD8-to-CD4 conversion, despite possible interaction of the CD8 T cells with MHCII in the thymus prior to adoptive transfer (Figure 3E).

In addition, we examined whether the immunocompromised status of the monoclonal TCR-transgenic model was required for the cross-differentiation. We reconstituted $OT1^+Rag^o$ neonates with splenocytes from normal B6 mice. The reconstitution with a large number of polyclonal immune cells did not preclude cross-differentiation of the OT1 clone to CD4 T cells, although it reduced the population size of the converted cells, possibly due to limitation of their expansion as a result of pre-occupancy of “niche” by the large population of cells transferred into the neonate recipients (Figure S4B–C).

Critical but non-essential role for microbiota in CD8-to-CD4 cross-differentiation

To determine whether normal microbial flora triggered the CD8 T-cell lineage plasticity in the large-intestine-associated microenvironment, we re-derived $OT1^+Rag^o$ mice under germ-free conditions, and then analyzed germ-free $OT1^+Rag^o$ mice. As shown in Figure 4A–B, $CD4^+Foxp3^-$ or $CD4^+Foxp3^+$ populations were diminished in LILP of the germ-free animals. However, germ-free conditions did not abrogate the cross-differentiated $CD4^+Foxp3^-$ or $CD4^+Foxp3^+$ populations in MLN of the $OT1^+Rag^o$ mice. Thus, the cross-differentiation did not require microbiota, but microbiota possibly promote homing to and / or expansion in LILP for the converted $CD4^+Foxp3^-$ or $CD4^+Foxp3^+$ T cells.

Furthermore, a defined microbial flora consisting of the gram-positive *Bacillus circulans* was sufficient to restore the $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ populations in LILP of $OT1^+Rag^o$ mice (Figure 4A–B). In addition, we tested whether the CD8-to-CD4 cross-differentiation depended on a particular facility or diet. The animals were raised in different buildings in combination with different diets. The cross differentiation of the OT1 T-cell clone to $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells occurred efficiently in the large-intestine-associated microenvironment in all variations of animal housing and diet we have examined (Figure S5).

Minimal role for T-cell-intrinsic TGFβ signaling in CD8-to-CD4 cross-differentiation

One of the major factors that influence T-cell differentiation in GAE is TGFβ (Li and Flavell, 2008). We tested whether TGFβ-mediated intrinsic signaling in T cells impacts the cross-differentiation from the CD8 lineage to CD4 T cells. We crossed $OT1^+Rag^o$ mice with a transgenic line carrying a dominant-negative mutant of TGFβ receptors, CD4-dnTGFBRII (Gorelik and Flavell, 2000). Blocking TGFβ signaling in T cells did not suppress cross-differentiation of the OT1 clone to CD4 T cells. It substantially reduced $CD4^+Foxp3^+$ population in LILP (Figure 4C), but not in lymphoid organs of $OT1^+Rag^o$ mice. Notably, it increased $CD4^+$ and $CD4^+Foxp3^+$ populations in the spleen (Figure 4D), an effect that remains to be explained.

Conversion from lineage-marked mature CD8 T cells with a physiological repertoire

To study whether mature T cells in the CD8 lineage can convert to CD4 T cells, we used the CD8aCre (Maekawa et al., 2008) × Rosa26YFP (Srinivas et al., 2001) model, in which the Cre-transgene expression driven by an engineered CD8 promoter was demonstrated to mark mature CD8 T cells in the periphery (Maekawa et al., 2008). In the $OT1^+Rag^oCD8aCre^+Rosa26YFP^+$ mice, $CD8aCre^+Rosa26YFP^+$ only marked a minority of CD8 T cells in the periphery (Figure S6A). $CD8^+YFP^+$ OT1 cells were purified from the peripheral lymphoid organs of $OT1^+Rag^oCD8aCre^+Rosa26YFP^+$ mice and transferred into Rag^o recipients. Indeed, the transferred $CD8^+YFP^+$ OT1 cells converted to CD4 T cells (Figure 5A), consistent with the results from adoptive transfer experiments using $CD8^+$ OT1 cells from $OT1^+Rag^o$ donors, as described before (Figure 3D).

We further examined cross-differentiation in a non-TCR transgenic setting. To unequivocally demonstrate that mature T cells in the CD8 lineage with a natural repertoire can indeed convert to CD4 T cells, we purified peripheral $CD8^+YFP^+CD4^-Foxp3^-CD25^-$ cells from $CD8aCre^+Rosa26YFP^+$ mice on the B6 genetic background, and adoptively transferred the cells into Rag^o/B6 recipients. The mature $CD8aCre^+Rosa26YFP^+$ cells converted to $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells in the new hosts, with high frequencies of CD4 T cells in LILP (average ~20%) followed by in MLN (Figure 5B). In unperturbed $CD8aCre^+Rosa26YFP^+$ mice, $CD4^+YFP^+$ cells existed rarely (Figure S6B). Depletion of the CD4 T-cell population enhanced cross-differentiation from the CD8 lineage to CD4 T cells (Figure 5C). Although these experiments had the caveats of the artificial promoter constructed to mark mature CD8 T cells in the periphery (Maekawa et al., 2008), and unknown antigen-specificity of transferred polyclonal CD8 T cells, the results indicate that naturally matured T cells in the CD8 lineage can indeed convert to CD4 T cells in the large-intestine-associated microenvironment.

A physiological role of CI-T_{reg} cells in selfless tolerance induction

Foxp3 expression in the $OT1^+Rag^o$ mice, identified with the Foxp3-IRES-RFP reporter (Wan and Flavell, 2005) or anti-Foxp3 antibodies, was only detected in the CD4 T-cell population but not in the CD8 T cells (Figure S7). To examine the physiological role of the $CD4^+Foxp3^+$ CI-T_{reg} cells in immune tolerance and homeostasis, we generated Foxp3-deficient $OT1^+Rag^o$ mice, by crossing $OT1^+Rag^o$ mice with the $Foxp3^{sf}$ line with a null

mutation of the *Foxp3* gene (Wildin et al., 2001). The Foxp3^{sf} line is a commonly used model for studying the role of Foxp3^{+} T_{reg} cells in their natural settings.

The OT1^{+}Rag^{o}Foxp3^{sf} mice developed healthily like OT1^{+}Rag^{o}Foxp3^{+} controls. Importantly, a functional *Foxp3* gene was not required for the cross-differentiation from the CD8 lineage to CD4 T cells (Figure S7C). However, CD8 T cells in the OT1^{+}Rag^{o}Foxp3^{sf} mice, when compared to the controls, exhibited an age-dependent increase in total counts and activation status in lymphoid organs (Figure 6A). These results indicate that the CD4^{+}Foxp3^{+} T cells converted from the CD8 lineage have a role as T_{reg} cells in their natural settings to regulate homeostasis of the conventional T-cell compartments.

Despite the accumulation of activated CD8 T cells in the OT1^{+}Rag^{o}Foxp3^{sf} mice, the animals exhibited growth and weight gains comparable to that of OT1^{+}Rag^{o}Foxp3^{+} controls. In consistency, pathology examination did not reveal substantial damage of the intestine tissues at the interface of the large intestine and microbiota in the OT1^{+}Rag^{o}Foxp3^{sf} mice (Figure 6B, left, top panels). Therefore, the CD4^{+}Foxp3^{+} CI-T_{reg} cells did not appear to have a role in tolerance induction to the constituents in the large intestine including microbiota, in contrast to other types of thymic or induced colonic T_{reg} cells (Cebula et al., 2013; Josefowicz et al., 2012; Lathrop et al., 2011).

We then examined if CD4^{+}Foxp3^{+} CI-T_{reg} cells protect the large intestine from inflammatory assault. We used a mouse model of colitis that was induced by oral administration of dextran sodium sulfate (DSS) (Okayasu et al., 1990). This is a commonly used model, in which the colitis was not caused by specific immune targeting, but due to chemically (DSS) induced inflammatory damage. DSS treatment induced colitis in both OT1^{+}Rag^{o}Foxp3^{sf} mice and OT1^{+}Rag^{o}Foxp3^{+} controls. After termination of the DSS treatment, OT1^{+}Rag^{o}Foxp3^{+} controls quickly recovered and there was no mortality in this group. However, OT1^{+}Rag^{o}Foxp3^{sf} mice could not heal from the colitis after discontinuing the DSS treatment and all animals in this group progressed to moribund conditions (Figure 6B). Thus, the CD4^{+}Foxp3^{+} CI-T_{reg} cells in their natural settings, specific to neither “self” nor microbial agents, not only regulated systemic immune homeostasis but also facilitated inflammation control in the large intestine.

A protective role of adoptively transferred CI-T_{reg} cells in T-cell-mediated immune damage

We then examined the regulatory potential of CI-T_{reg} cells in an adoptive transfer setting. Led by the findings shown in Figure 6 that CI-T_{reg} cells reduced activation of CD8 T cells, we used a model of colitis induced by transfer of naïve CD8 effector T (T_{eff}) cells into Rag^{o} recipients (Tajima et al., 2008). OT1 CD4^{+}Foxp3^{+} CI-T_{reg} cells co-transferred at 1:3 ratio with the CD8 T_{eff} cells indeed suppressed the activation of the CD8 T_{eff} cells (Figure 7A–B), improved weight maintenance (Figure 7C) and suppressed colitis development (Figure 7D). Co-transfer of OT1 CD4^{+}Foxp3^{-} cells did not suppress but slightly increased the activation of CD8 T_{eff} cells. It did not inhibit colitis development. Of note, in the OT1 CD4^{+}Foxp3^{-} co-transfer group, ~30% of OT1 CD4 cells became Foxp3^{+} in the new host (not shown). The CI-T_{reg} cells appeared to be more consistent than standard CD4^{+}Foxp3^{+} T_{reg} cells from B6 mice in suppressing immune damage in this model (Figure 7C–D). Further experimentations at a larger scale and with various cell doses are needed to test if

CI-T_{reg} cells might be more effective than standard $CD4^+Foxp3^+$ T_{reg} cells in this and other conditions of CD8 T-cell-mediated damage. Future studies are also needed to examine whether CI-T_{reg} cells function in other settings of inflammatory damage, including immune damage mediated by CD4 T_{eff} cells. Taken together, the results from Figure 6 and Figure 7 suggest that CI-T_{reg} cells can indeed be effective in suppressing inflammatory damage to intestinal tissue.

DISCUSSION

Originally formulated to explain the biological relationship of a vertebrate host organism and microbial pathogens, the concept of selfhood-centered tolerance versus immunity has led to much of immunological understandings. Its limitations (Matzinger, 1994) have been highlighted with the recent debates of immune tolerance induction to microbiota in the large intestine (Cebula et al., 2013; Josefowicz et al., 2012; Lathrop et al., 2011). A healthy mammal typically hosts a chimeric composition of cells from diverse genetic origins. The steady-state symbiosis with a superior number of normal flora microbial agents in the large intestine compels a reconsideration of the concept of “self” and “nonself” discrimination, as the mutualistic microbial organisms in a mammalian host do not fit into the traditional definition of “self” or “nonself”.

This study demonstrates conversion of T-cell clones in the CD8 lineage to $CD4^+Foxp3^-$ and $CD4^+$ $Foxp3^+$ T_{reg} cells in the large-intestine-associated microenvironment, in the absence of its nominal antigen, or despite the remote presence of a “self” antigen in a distal organ. This observation suggests that, in a steady state without apparent “danger” (Matzinger, 1994), T cells do not necessarily delineate a dichotomized discrimination of “self” from “nonself”, but can differentiate with “selfless”, intrinsic plasticity to remedy an imbalanced constitution. Conversion of the CD8 T-cell lineage in the large-intestine-associated microenvironment could be driven by environmental cues without regard to the “self” or “nonself” origin of specific antigenic signals. In this regard, although microbial-specific tolerance could indeed be detected in the large intestine (Cebula et al., 2013; Lathrop et al., 2011), the effect of gut flora on T-cell differentiation may not always be limited to the gut. For example, CD4 Th17 subset differentiation induced by gut-microbial flora could trigger autoimmune damage in the joint, a site distal from the gut (Wu et al., 2010).

The two independent T-cell clones in the MHC-class-I-restricted CD8 lineage showed lineage plasticity in the large-intestine-associated microenvironment. This plasticity may be T-cell intrinsic, since it was not apparently induced by antigenic stimulation from microbiota or the body’s self. To an extent, the observation offers some reconciliation between “selfhood”-centered immunological discrimination and the chimeric composition of a healthy mammal. In this regard, we suggest distinction between central and peripheral T-cell development. Robust evidence exists for T-cell clonal selection based on “self / nonself” discrimination in a central lymphoid organ, the thymus (Anderson et al., 2002; Hsieh et al., 2004; Kisielow et al., 1988). However, in the periphery, in addition to the various mechanisms of tolerance induction to self-antigens, T-cell differentiation at a heterogeneous interface such as GAE could occur independently of “self”-denotation (Matzinger, 1994), in a “selfless” mode of clonal plasticity. Such kind of plasticity may facilitate tolerance and

integrity with respect to a wholesome biological entity of a mammalian organism. In other words, there is an additional, peripheral mechanism (s) of T-cell immunity versus tolerance induction that may occur regardless of “self” or “nonself”, but is attuned to environmental cues and evolved for a systemic balance, with an intrinsic cellular plasticity equipped in the CD8 T-cell lineage.

Technically, it is a challenge to study T-cell clonal fate in a natural history of peripheral development without resorting to a reductionist approach, particularly in complex settings such as the large-intestine-associated microenvironment. On the other hand, how microbiota affects the clonal fate of T lymphocytes remains a question of extensive interest. This study used monoclonal TCR transgenic models that made it possible to analyze the T-cell clonal fate in terms of known antigen-specificities. The nature of such *in vivo* studies limited the practicality to only a few clones. Of note, the $OT1^{+}Rag^{o}$ mouse is a standard model generally used for studying CD8 T cells. Still, the limitation of such an approach is apparent: how physiologically relevant is such an exaggerated simplicity of T-cell repertoire in a context of relative lymphopenia?

A previous study showed that a well-established T-cell lineage such as the CD4 T_{reg} cell was stable in lineage specification even during lymphopenia-driven proliferation (Rubtsov et al., 2010). In our studies, lymphopenia was unlikely the original trigger for the CD8-to-CD4 lineage plasticity either. Rectifying the monoclonal and lymphopenic condition by neonatal reconstitution with a natural repertoire of immune cells did not preclude the cross-differentiation, but reduced the size of converted populations, suggesting that the CD8-to-CD4 cross-differentiation may occur at a basal level, and its expansion depends on available “niche”. Consistent with this possibility, the CD8-to-CD4 conversion of polyclonal T cells in $CD8aCre^{+}Rosa26YFP^{+}$ mice was enhanced in CD4-depleted animals. Of note, the CD8aCre transgene was constructed with an unnatural promoter (Maekawa et al., 2008). Although it labeled most of the peripheral CD8 T cells in B6 mice, it only marked a minority of OT1 CD8 T cells in peripheral lymphoid organs. Therefore, an accurate assessment of the extent of CD8-to-CD4 lineage cross-differentiation in a natural repertoire awaits an alternative tool for efficient lineage tracing unaffected by clonal variations. Nevertheless, CD8-to-CD4 lineage conversion revealed in this study could be relevant in adoptive immune therapies using CD8 T cells, or in clinical settings of immune imbalance due to CD4 T-cell deficit, such as AIDS or immunoablation conditioning.

It remains to be determined whether the $CD4^{+}Foxp3^{-}$ and $CD4^{+}Foxp3^{+}$ cells derived from cross-differentiation of the CD8 lineage have a stable lineage specification or merely reflect a metastable differentiation state. Lineage stability in various settings has been demonstrated for MHC-class-II-restricted $CD4^{+}Foxp3^{+}$ T_{reg} cells (Rubtsov et al., 2010). In our study, the functional capacity of $CD4^{+}Foxp3^{+}$ CI-T_{reg} cells was indeed evident *in vivo*, but their lineage stability needs to be characterized by genetic tracing. With regard to their therapeutic potentials, unlike the known subsets of thymic and peripheral $CD4^{+}Foxp3^{+}$ T_{reg} cells which recognize MHC-class-II molecules, expression of which is limited to antigen-presenting cells, the $CD4^{+}Foxp3^{+}$ CI-T_{reg} cells are distinct in recognizing constitutively and ubiquitously expressed MHC-class-I molecules. Therefore, the “selfless” plasticity of the

CD8 lineage cross-differentiating to $CD4^+Foxp3^+$ CI-T_{reg} cells suggests novel possibilities for immune tolerance induction.

EXPERIMENTAL PROCEDURES

Mice

The transgenic and knockout mouse models were described previously: OT1 (Hogquist et al., 1994), 8.3 (Verdaguer et al., 1997), OTII (Barnden et al., 1998), BDC2.5 (Katz et al., 1993), $Rag1^o$ (Mombaerts et al., 1992), $Foxp3^{sf}$ (Wildin et al., 2001), MHC class II H-2Ab knockout (Grusby et al., 1991), MHC-class-II KO (Madsen et al., 1999), $Foxp3^{FIR}$ (Foxp3-IRIS-RFP "knockin") mice (Wan and Flavell, 2005), CD8aCre (Maekawa et al., 2008), Rosa-26-YFP (Srinivas et al., 2001), TGFβ1 Receptor dominant negative (DN) transgenic mice (Gorelik and Flavell, 2000). These lines were crossed in various combinations to generate the model for this study. Germ-free (**GF**) mice were re-derived by embryo transfer at Taconic (Hudson, NY). During germ-free re-derivation of $OT1^+Rag^oFoxp3^{FIR}$ mice, one of the isolators was found to be specific-pathogen-free but an aerobic normal microbial flora was detected during monitoring. The flora was identified as gram-positive *Bacillus circulans*. The animals from this isolator, although of an unsuccessful attempt in germ-free re-derivation, were deemed with a "defined flora" (**DF**), and analyzed in comparison to animals that are maintained in standard specific-pathogen-free facility (SPF) with normal flora (**NF**) or germ-free (**GF**) conditions. Other animals were maintained in a specific-pathogen-free (**SPF**) barrier facility, with diet Teklad #2018 or #2019 (Harlan Laboratories, Madison, WI), or LabDiet diet #5021(LabDiet, St. Louis, MO). The studies are approved by the Institutional Animal Care and Use Committee at the University of Miami.

Surgical removal of mesenteric lymph nodes and thymectomy

Mock surgery or surgical removal of MLN were conducted on 13- to 14-day old $OT1^+Rag^o$ mice with a procedure similar to previously reported (Griffin et al., 2011). Thymectomy was conducted according to a standard procedure. Both are described in detail in the SUPPLEMENTAL EXPERIMENTAL PROCEDURES.

Antibody depletion of CD4 T cells

For CD4 T cell depletion, mice were injected i.p. with anti-CD4 antibody (GK1.5) or Rat IgG controls, 12 μg/g weight, at day 0 and 5. Efficacy of anti-CD4 T cell depletion was verified by tail vein blood sampling after completion of treatment. The mice were then monitored for CD4 T-cell population recovery. The end point experiments were conducted 35–40 weeks after completion of anti-CD4 depletion.

Cell preparation from intestinal tissue

Intestines were minced and treated with EDTA to isolate IELs. Collagenase was used to dissociate intestinal tissues for lymphocyte isolation from lamina propria. Detailed methods were described in the SUPPLEMENTAL EXPERIMENTAL PROCEDURES.

Flow cytometry, cell sorting and adoptive transfer experiments

Flow cytometry analyses, including intracellular cytokine staining, were conducted with a standard procedure (Devarajan et al., 2014; Miska et al., 2014; Miska et al., 2012), described in the Supplemental Procedures. In adoptive transfer experiments, CD8 T cells were sorted as $CD8^+CD4^-CD11b^-CD11c^-Ter119^-B220^-NK1.1^-CD25^-Foxp3^-$ from the spleen and lymph nodes of $OT1^+Rag^oFoxp3^{FIR+}$ mice or as $CD8^+YFP^+CD4^-CD11b^-CD11c^-Ter119^-B220^-NK1.1^-CD25^-Foxp3^-$ from $CD8aCre^+$ $Rosa26YFP^+$ mice. The purified CD8 T cells (purity > 99.7%), with or without the YFP marker, were injected into recipients. For neonate reconstitution of $OT1^+Rag^oFoxp3^{FIR+}$ mice, whole splenocyte preparation from B6 mice were injected, 2×10^7 cells per recipient, by intraperitoneal injection.

Colitis studies

Colitis induction by DSS was done following previously described methods (Okayasu et al., 1990), which are described in details in the Supplemental Procedure. Colitis induction by transfer of purified naïve CD8 effector T (T_{eff}) cells into Rag^o recipients and pathology scoring were done as described before (Tajima et al., 2008) except a lower dose of CD8 T cells were used.

Statistics

*S*tudent's *t* tests or Mann-Whitney *U* tests were used when appropriate for single comparisons. For multiple groups, ANOVA analyses followed up with Tukey's post-hoc test or Mann-Whitney *U* tests followed by Bonferroni's correction were used. For survival analysis, log-rank test was conducted. P values equal to or less than 0.05 were considered significant. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, Paley MA, Antenus M, Williams KL, Erikson J, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity. 2012; 37:158–170. [PubMed: 22705104]

Anderson MK. Changing course by lymphocyte lineage redirection. Nat Immunol. 2013; 14:199–201. [PubMed: 23416670]

Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, von Boehmer H, Bronson R, Dierich A, Benoist C, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science. 2002; 298:1395–1401. [PubMed: 12376594]

Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol. 1998; 76:34–40. [PubMed: 9553774]

Burnet FM. A modification of Jerne's theory of antibody production using the concept of clonal selection. Aust J Sci. 1957; 20:67–69.

Cebula A, Seweryn M, Rempala GA, Pabla SS, McIndoe RA, Denning TL, Bry L, Kraj P, Kisielow P, Ignatowicz L. Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. Nature. 2013; 497:258–262. [PubMed: 23624374]

Devarajan P, Miska J, Lui JB, Swieboda D, Chen Z. Opposing Effects of CTLA4 Insufficiency on Regulatory versus Conventional T Cells in Autoimmunity Converge on Effector Memory in Target Tissue. J Immunol. 2014; 193:4368–4380. [PubMed: 25246499]

Doyle C, Strominger JL. Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature. 1987; 330:256–259. [PubMed: 2823150]

Esplugues E, Huber S, Gagliani N, Hauser AE, Town T, Wan YY, O'Connor W Jr, Rongvaux A, Van Rooijen N, Haberman AM, et al. Control of TH17 cells occurs in the small intestine. Nature. 2011; 475:514–518. [PubMed: 21765430]

Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003; 4:330–336. [PubMed: 12612578]

Gorelik L, Flavell RA. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity. 2000; 12:171–181. [PubMed: 10714683]

Griffin AJ, Li LX, Voedisch S, Pabst O, McSorley SJ. Dissemination of persistent intestinal bacteria via the mesenteric lymph nodes causes typhoid relapse. Infect Immun. 2011; 79:1479–1488. [PubMed: 21263018]

Grusby MJ, Johnson RS, Papaioannou VE, Glimcher LH. Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. Science. 1991; 253:1417–1420. [PubMed: 1910207]

Hedrick SM. Positive selection in the thymus: an enigma wrapped in a mystery. J Immunol. 2012; 188:2043–2045. [PubMed: 22345700]

Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. Cell. 1994; 76:17–27. [PubMed: 8287475]

Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol. 2010; 10:159–169. [PubMed: 20182457]

Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003; 299:1057–1061. [PubMed: 12522256]

Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, Rudensky AY. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. Immunity. 2004; 21:267–277. [PubMed: 15308106]

Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009; 139:485–498. [PubMed: 19836068]

Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002; 20:197–216. [PubMed: 11861602]

Josefowicz SZ, Niec RE, Kim HY, Treuting P, Chinen T, Zheng Y, Umetsu DT, Rudensky AY. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature. 2012; 482:395–399. [PubMed: 22318520]

Katz JD, Wang B, Haskins K, Benoist C, Mathis D. Following a diabetogenic T cell from genesis through pathogenesis. Cell. 1993; 74:1089–1100. [PubMed: 8402882]

Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol. 2003; 4:337–342. [PubMed: 12612581]

Kirchner J, Bevan MJ. ITM2A is induced during thymocyte selection and T cell activation and causes downregulation of CD8 when overexpressed in CD4(+)CD8(+) double positive thymocytes. J Exp Med. 1999; 190:217–228. [PubMed: 10432285]

Kisielow P, Bluthmann H, Staerz UD, Steinmetz M, von Boehmer H. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. Nature. 1988; 333:742–746. [PubMed: 3260350]

Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS. Peripheral education of the immune system by colonic commensal microbiota. Nature. 2011; 478:250–254. [PubMed: 21937990]

Lee JW, Epardaud M, Sun J, Becker JE, Cheng AC, Yonekura AR, Heath JK, Turley SJ. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. Nat Immunol. 2007; 8:181–190. [PubMed: 17195844]

Li MO, Flavell RA. TGF-beta: a master of all T cell trades. Cell. 2008; 134:392–404. [PubMed: 18692464]

Madsen L, Labrecque N, Engberg J, Dierich A, Svejgaard A, Benoist C, Mathis D, Fugger L. Mice lacking all conventional MHC class II genes. Proc Natl Acad Sci U S A. 1999; 96:10338–10343. [PubMed: 10468609]

Maekawa Y, Minato Y, Ishifune C, Kurihara T, Kitamura A, Kojima H, Yagita H, Sakata-Yanagimoto M, Saito T, Taniuchi I, et al. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. Nat Immunol. 2008; 9:1140–1147. [PubMed: 18724371]

Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol. 1994; 12:991–1045. [PubMed: 8011301]

Miska J, Abdulreda MH, Devarajan P, Lui JB, Suzuki J, Pileggi A, Berggren PO, Chen Z. Real-time immune cell interactions in target tissue during autoimmune-induced damage and graft tolerance. J Exp Med. 2014; 211:441–456. [PubMed: 24567447]

Miska J, Bas E, Devarajan P, Chen Z. Autoimmunity-mediated antitumor immunity: tumor as an immunoprivileged self. Eur J Immunol. 2012; 42:2584–2596. [PubMed: 22777737]

Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell. 1992; 68:869–877. [PubMed: 1547488]

Mucida D, Husain MM, Muroi S, van Wijk F, Shinnakasu R, Naoe Y, Reis BS, Huang Y, Lambolez F, Docherty M, et al. Transcriptional reprogramming of mature CD4(+) helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes. Nat Immunol. 2013; 14:281–289. [PubMed: 23334788]

Nehls M, Pfeifer D, Schorpp M, Hedrich H, Boehm T. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. Nature. 1994; 372:103–107. [PubMed: 7969402]

Norment AM, Salter RD, Parham P, Engelhard VH, Littman DR. Cell-cell adhesion mediated by CD8 and MHC class I molecules. Nature. 1988; 336:79–81. [PubMed: 3263576]

Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology. 1990; 98:694–702. [PubMed: 1688816]

Reis BS, Rogoz A, Costa-Pinto FA, Taniuchi I, Mucida D. Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4(+) T cell immunity. Nat Immunol. 2013; 14:271–280. [PubMed: 23334789]

Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, Benoist C, Rudensky AY. Stability of the regulatory T cell lineage in vivo. Science. 2010; 329:1667–1671. [PubMed: 20929851]

Rudd CE, Trevillyan JM, Dasgupta JD, Wong LL, Schlossman SF. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc Natl Acad Sci U S A. 1988; 85:5190–5194. [PubMed: 2455897]

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol. 2001; 1:4. [PubMed: 11299042]

Tajima M, Wakita D, Noguchi D, Chamoto K, Yue Z, Fugo K, Ishigame H, Iwakura Y, Kitamura H, Nishimura T. IL-6-dependent spontaneous proliferation is required for the induction of colitogenic IL-17-producing CD8+ T cells. J Exp Med. 2008; 205:1019–1027. [PubMed: 18426983]

Veillette A, Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. Cell. 1988; 55:301–308. [PubMed: 3262426]

Verdaguer J, Schmidt D, Amrani A, Anderson B, Averill N, Santamaria P. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. J Exp Med. 1997; 186:1663–1676. [PubMed: 9362527]

Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl Acad Sci U S A. 2005; 102:5126–5131. [PubMed: 15795373]

Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet. 2001; 27:18–20. [PubMed: 11137992]

Wu HJ, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, Littman DR, Benoist C, Mathis D. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010; 32:815–827. [PubMed: 20620945]

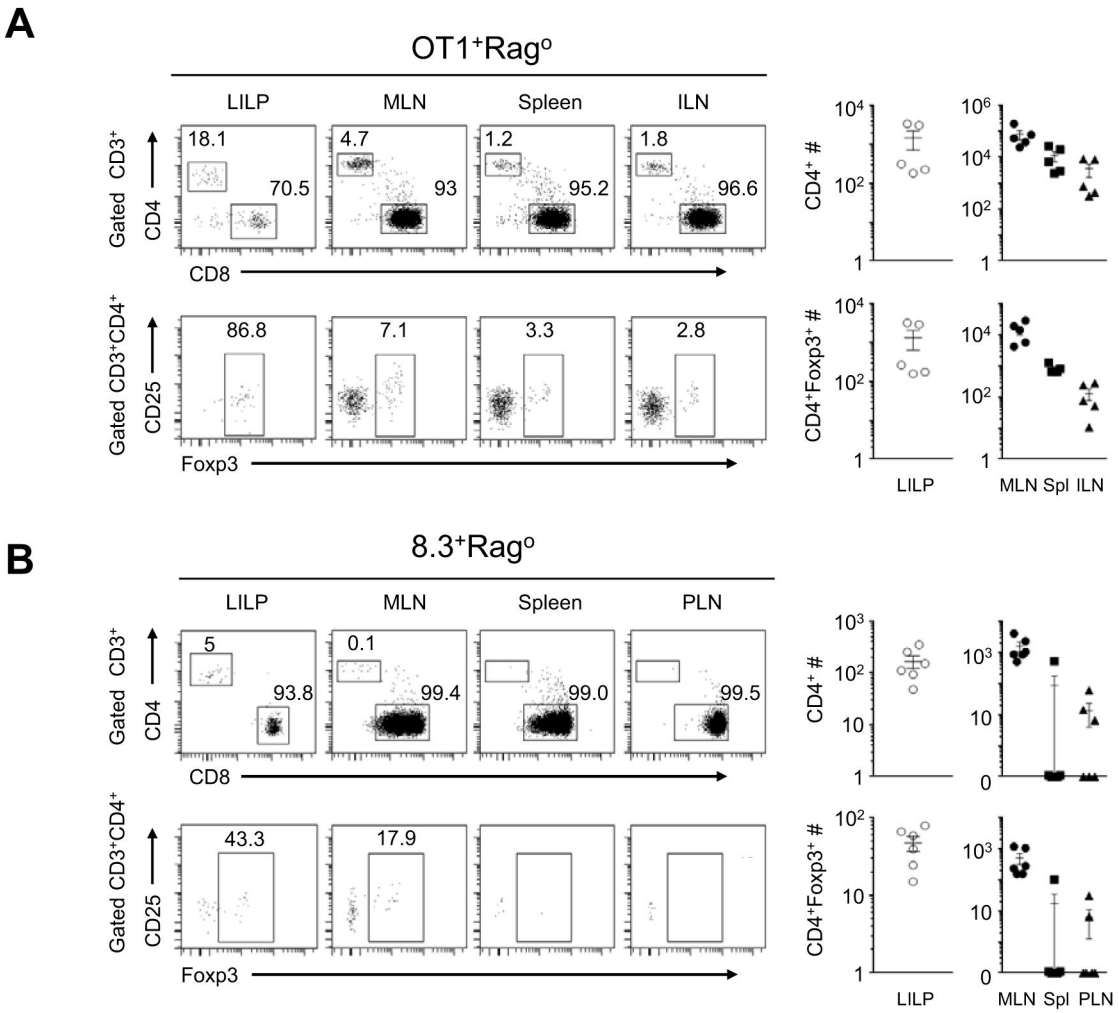

Figure 1. Cross-differentiation of two independent clones in the MHC-class-I-restricted CD8 T-cell lineage to $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells

(A) Representative flow cytometry of the large intestinal lamina propria (LILP), mesenteric lymph nodes (MLN), spleen and inguinal lymph nodes (ILN) in 5-week old $OT1^+Rag^o$ mice, followed by total cell counts for $CD4^+$ and $CD4^+$ $Foxp3^+$ T cells (n=5, mean±SEM). (**B**) Representative flow cytometry of LILP, MLN, spleen and pancreatic lymph nodes (PLN) of 7-wk-old NOD/B6.8.3$^+$Rag^o mice, followed by $CD4^+$ and $CD4^+Foxp3^+$ cell numbers in different organs (n=6, mean±SEM). The numbers in the flow cytometry plots represent percentages of gated populations. Each data point represents one animal.

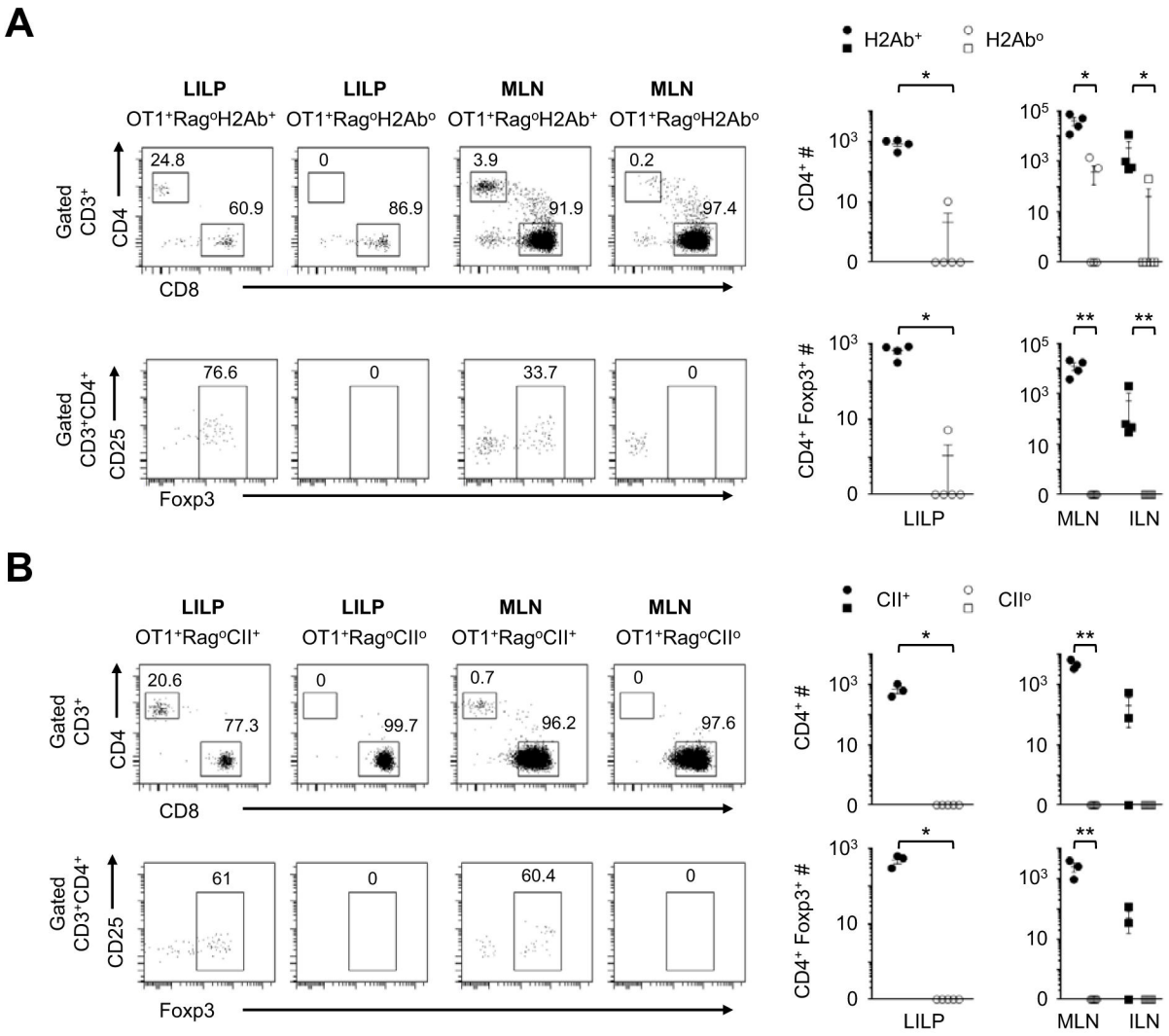

Figure 2. Cross-differentiation from the CD8 lineage to CD4 T cells with a requirement of MHC Class II

(A) OT1^{+}Rag^{o} B6 mice were crossed with the H-2Ab knockout line to generate H-2Ab-deficient OT1^{+}Rag^{o} mice (n=5) and controls (n=4). On the B6 genetic background, H-2Ab knockout effectively renders deficiencies in MHC class II. The animals were analyzed at 6 to 10 wks of age. Representative flow cytometry plots are followed with cell counts of CD4^{+} and CD4^{+}Foxp3^{+} T cells. Closed and open symbols denote H2-Ab^{+} controls and H2-Ab$^{−}$ mice, respectively. Each symbol represents one animal (mean±SEM). **(B)** OT1^{+}Rag^{o} mice were crossed with the knockout line of the whole MHC class II locus to generate class-II-deficient OT1^{+}Rag^{o} mice (n=5) and controls (n=3). The animals were analyzed at 4 to 21 wks of age. Representative flow cytometry plots are followed with cell counts of CD4^{+} and CD4^{+}Foxp3^{+} T cells. Closed and open symbols denote MHCII^{+} and MHCII$^{−}$ mice, respectively. Each data point represents one animal (mean±SEM). *, p<0.05; **, p<0.01.

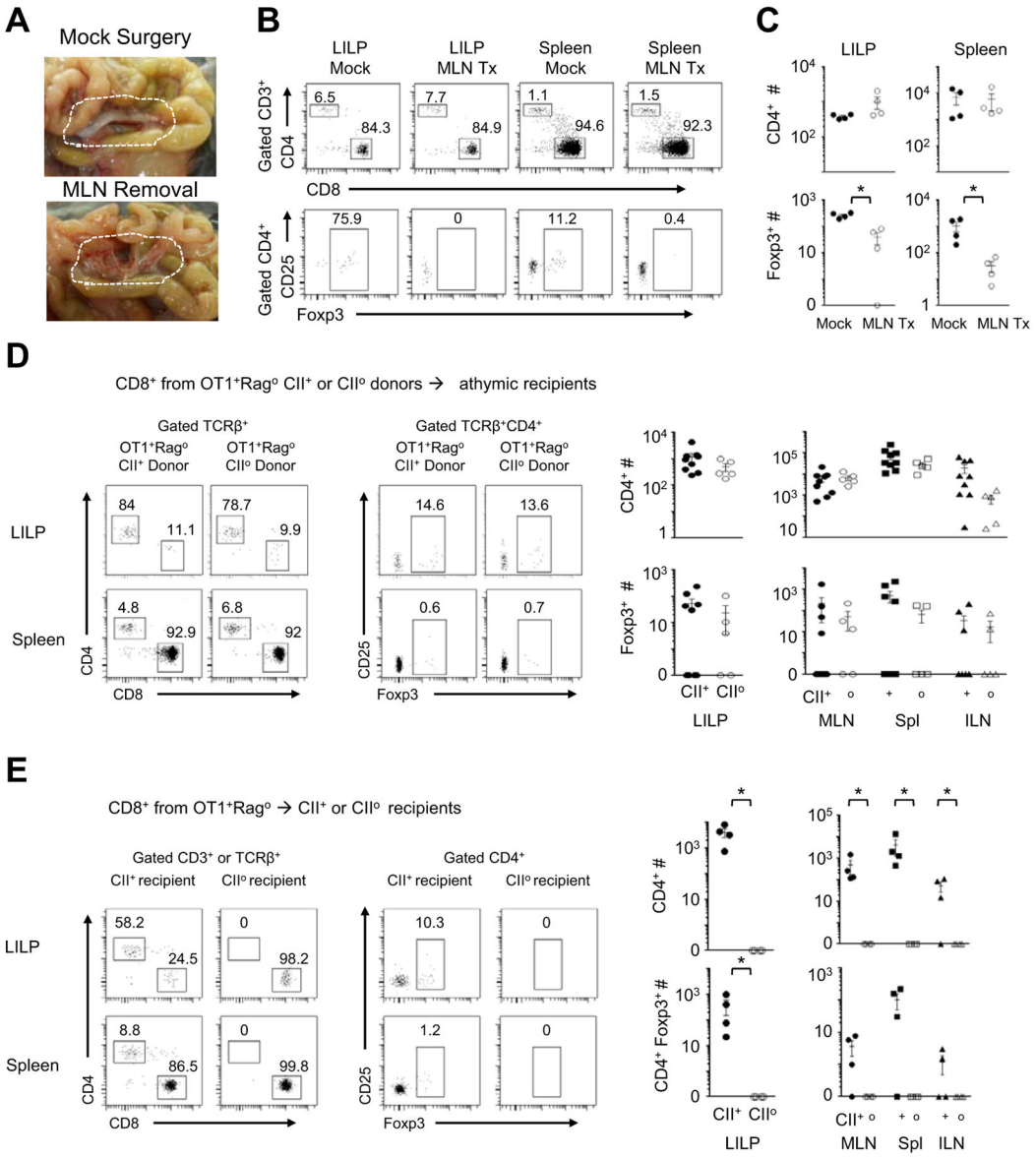

Figure 3. A role of MLN but not the thymus in cross-differentiation from the CD8 T-cell lineage to CD4$^+$Foxp3$^+$ CI-T$_{reg}$ cells

(**A–C**) Analyses of OT1$^+$Rag^o mice 2 wks post MLN removal: (**A**) success of MLN removal; (**B**) representative flow cytometry plots (the numbers in the flow cytometry plots represent percentages of gated populations); (**C**) Summary of CD4$^+$ and CD4$^+$Foxp3$^+$ cell counts (n=4, mean±SEM). (**D**) Conversion of purified peripheral OT1$^+$CD8$^+$CD4$^-$CD25$^-$Foxp3$^-$ cells after transfer into athymic hosts. MHCII$^+$ or MHCII^o OT1$^+$Rag^o donors were used to purify the peripheral CD8 T cells to examine whether presence of MHCII during maturation of the CD8 T cells enables a lasting potential for their conversion to CD4$^+$ cells in a new host. (**E**) MHCII$^+$ or MHCII^o Rag^o recipients were used to examine the requirements of MHCII during conversion of adoptively transferred OT1$^+$Rag^o CD8 T cells. Representative flow cytometry plots are followed by a summary of

$CD4^+$ and $CD4^+Foxp3^+$ cell counts. Each data point represents one animal (n=5–7, mean ±SEM).*, $p<0.05$.

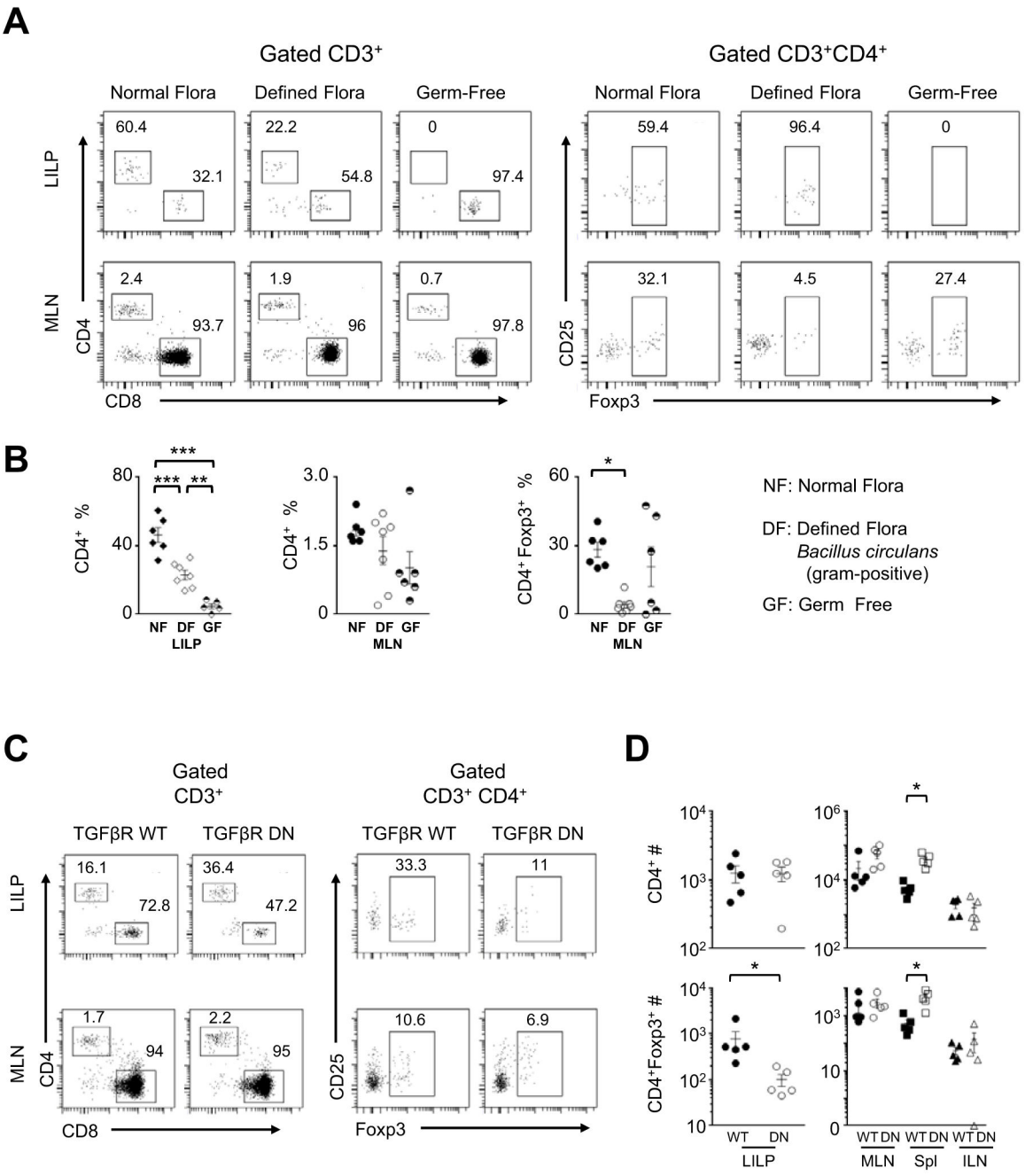

Figure 4. Roles of microbiota and TGFβ in the CD8-to-CD4 cross-differentiation

(**A**) Representative flow cytometry of OT1^{+}Rag^{o} mice from germ-free (**GF**) facility, compared to animals in specific pathogen-free facility with normal flora (**NF**), or with a defined flora (**DF**) of the gram-positive *Bacillus circulans*. (**B**) Summary of CD4^{+} and CD4^{+}Foxp3^{+} percentages in the OT1^{+}Rag^{o} mice under GF versus NF and DF conditions (n=5–7, mean±SEM). (**C–D**) OT1^{+}Rag^{o} mice were crossed with the CD4-dnTGFBRII transgenic line (TGFβR DN), to examine the effect of T-cell intrinsic TGFβ signaling in the cross-differentiation. Representative flow cytometry of OT1^{+}Rag^{o} TGFβR DN mice versus

$OT1^{+}Rag^{o}$ controls (**C**), followed by summary of cell counts (n=5, mean±SEM; closed and open symbols, control and TGFβR DN mice, respectively) (**D**). The numbers in the flow cytometry plots represent percentages of gated populations. Each data point represents one animal. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

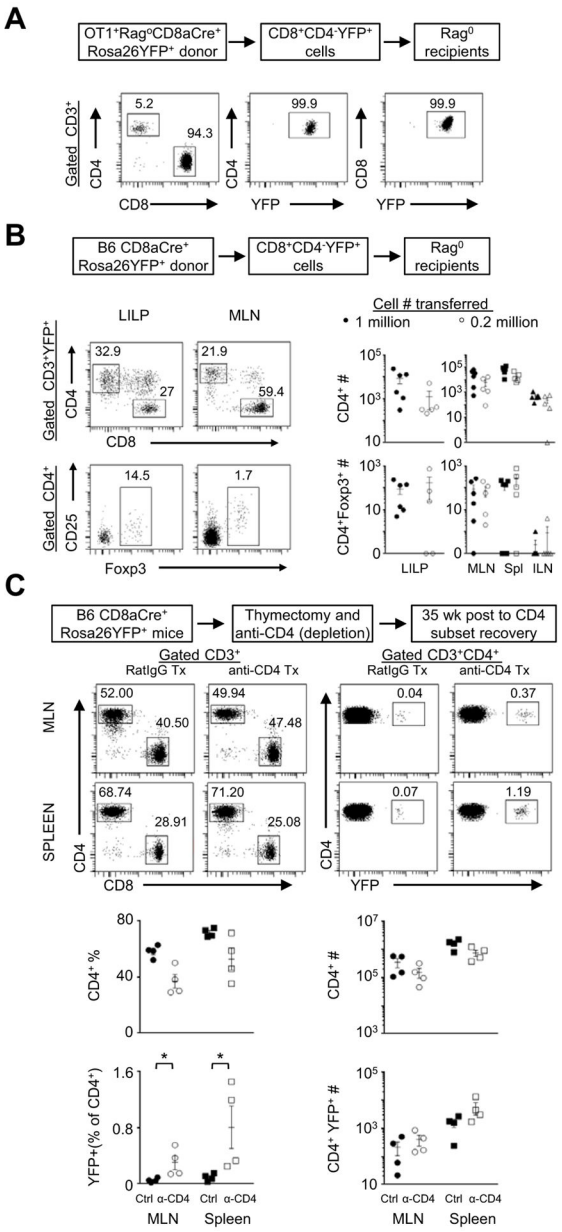

Figure 5. Conversion of lineage-marked CD8 T cells to CD4$^+$ and CD4$^+$Foxp3$^+$ T cells

(A) Conversion of lineage-marked OT1 CD8 T cells. CD8$^+$YFP$^+$CD4$^-$CD25$^-$ cells purified from OT1$^+$Rag^{o}CD8aCre$^+$Rosa26YFP$^+$ mice were transferred into Rag^o recipients and analyzed ~6wks later (n=4). Representative flow cytometry plots of MLN. The numbers in the plots indicate the percentages of gated populations. **(B) Conversion of lineage-marked polyclonal CD8 T cells.** CD8$^+$YFP$^+$CD4$^-$CD25$^-$Foxp3$^-$ cells were purified from CD8aCre$^+$Rosa26YFP$^+$ B6 mice and adoptively transferred into Rag^o hosts, each receiving 1.0×10^6 cells (closed symbols) or 2×10^5 cells (open symbols). The animals were analyzed ~6 wks later. Representative flow plots of T cells in LILP and MLN were followed by summary of CD4$^+$ and CD4$^+$Foxp3$^+$ cell counts. Each data point represents one animal (n=5–6, mean±SEM). **(C) Influence of CD4-depletion.** Adult B6 CD8aCre$^+$Rosa26YFP$^+$

mice were thymectomized (to eliminate thymic development). Two weeks later, mice were treated with two doses of Rat IgG controls or anti-CD4 depleting antibodies. Mice were analyzed by flow cytometry at 35–40 wks post treatment (after the CD4 T-cell population recovered from depletion). Representative flow cytometry plots are followed by summary of percentages and cell counts. Each data point represents one animal (mean±SEM, n=4–5). *, $p<0.05$.

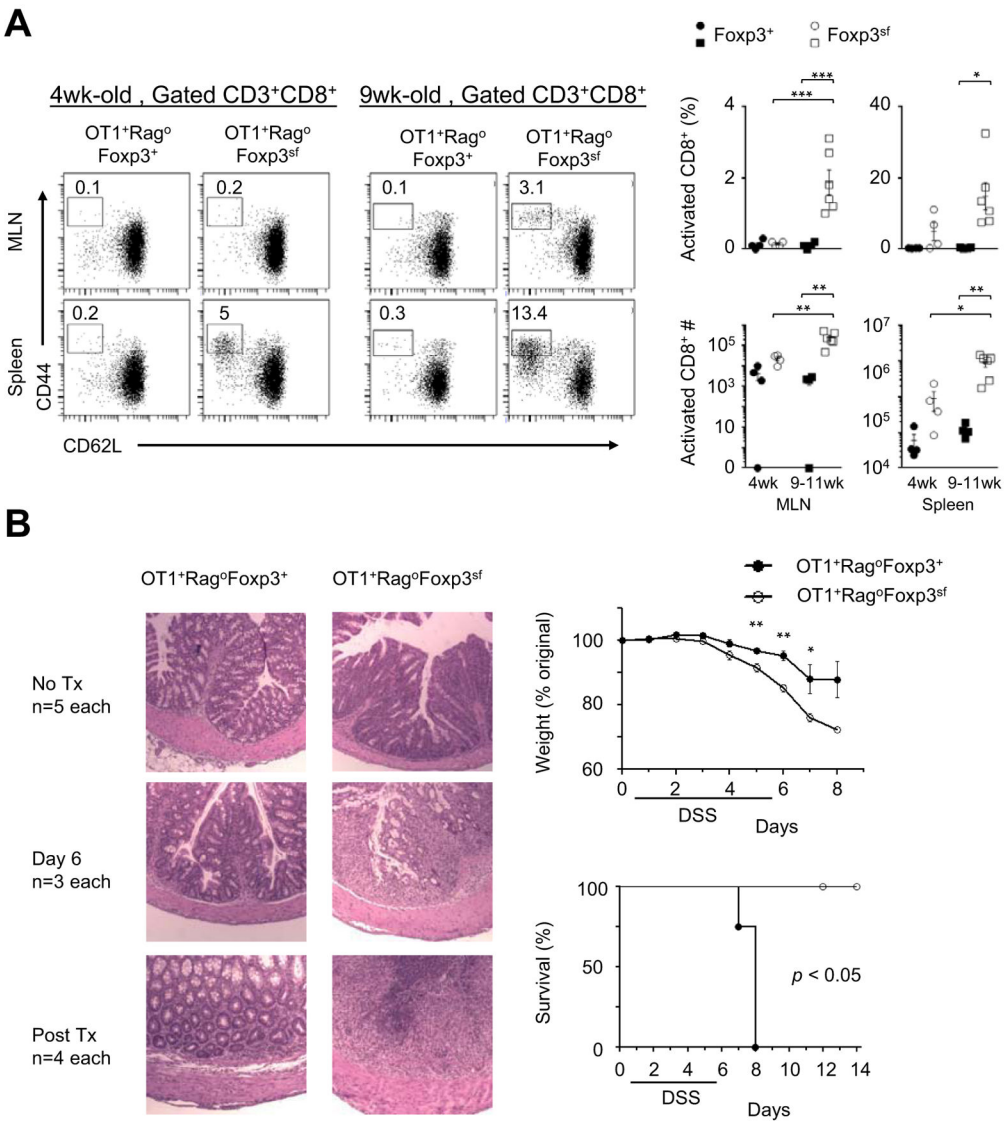

Figure 6. Function of CD4$^+$Foxp3$^+$ CI-T$_{reg}$ cells in their natural settings in regulating immune homeostasis and protecting the large intestine from inflammatory damage

(A) The effect of Foxp3-deficiencies in OT1$^+$Rag^o mice on homeostasis and activation of CD8 T cells. Representative flow cytometry followed by cell counts (closed symbols, OT1$^+$Rag^{o}Foxp3$^+$ mice; open symbols, OT1$^+$Rag^{o}Foxp^{sf} mice). The numbers in the flow cytometry plots represent percentages of gated populations. Each data point represents one animal (n=4–6; mean±SEM). (**B**) Colitis development in OT1$^+$Rag^{o}Foxp3$^+$ versus OT1$^+$Rag^{o}Foxp3^{sf} mice treated with DSS drinking water. Left, histopathology of colon tissue sections (original magnification × 5). Right, weight loss and survival curves (n=4–7 pooled from two experiments, mean±SEM). *, p<0.05; **, p<0.01; ***, p<0.001.

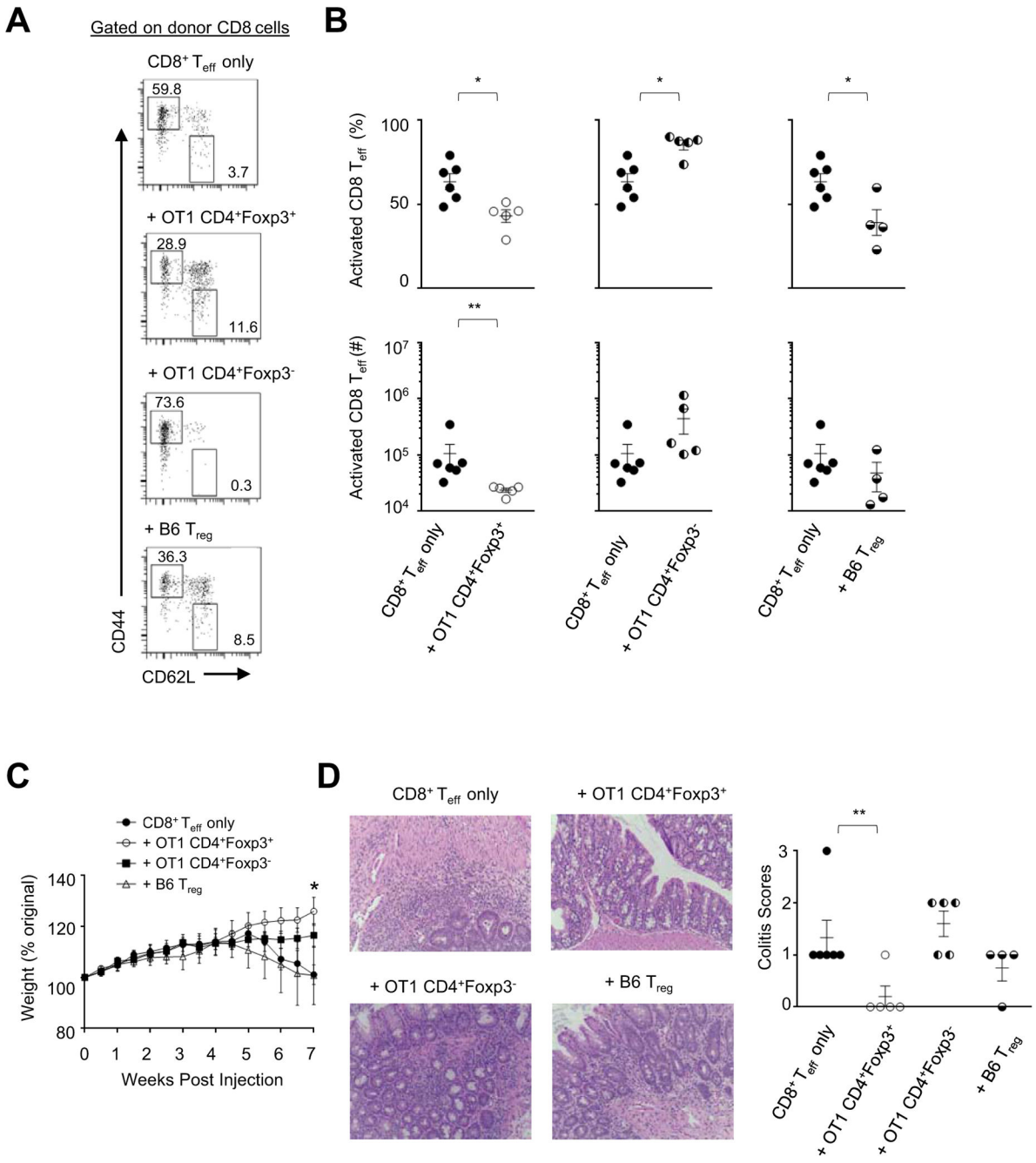

Figure 7. Control of T-cell-mediated damage by CD4$^+$Foxp3$^+$ CI-T$_{reg}$ cells in adoptive transferring settings

Purified naïve CD8 T_{eff} cells from wildtype B6 mice were adoptively transferred into Rag^o recipients. The CD8 T_{eff} cells (2×10^5) were injected alone, or co-transferred with 7×10^4 CD4$^+$Foxp3$^+$ or CD4$^+$Foxp3$^-$ cells purified from OT1$^+$Rag^o mice, or CD4$^+$Foxp3$^+$ cells from B6 mice. Mice were monitored for weight. **(A)** Representative flow cytometry. The numbers in the flow-cytometry plots represent percentages of gated populations. (**B**) Summary of percentages and cell counts for the activated CD8 T cells. Each data point represents one animal (n=4–6; mean±SEM). (**C**) Weight of the animals after T cell transfer.

(**D**) Pathology assessment of colitis development. Left, H–E staining of colon tissue sections (original magnification × 12.5). Right, histopathology score (mean±SEM; n=4–6 pooled from 4 experiments). *, $p<0.05$; **, $p<0.01$.