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Functional Plasticity of Antigen-Specific Regulatory T Cells in Context of Tumor

Caroline Addey,* Matthew White,* Lang Dou,* David Coe,* Julian Dyson,† and Jian-Guo Chai*

Although polyclonal regulatory T cells (Tregs) that once expressed Foxp3 (ex-Tregs) derived from Foxp3+ Tregs have been described in homeostatic and autoimmune settings, little is known regarding the influence of the tumor environment on ex-Treg development. After adoptive transfer of HY-specific green Tregs (peripheral or thymic) to Rag2−/− B6 female mice bearing syngeneic HY-expressing MB49 tumors, a significant fraction rapidly lost expression of Foxp3. On the second transfer to a Rag2−/− B6 male environment, these ex-Tregs expanded strongly, whereas Tregs that maintained expression of Foxp3 expression did not. Both FACS and quantitative real-time PCR analysis revealed that ex-Tregs upregulated genes characteristic of a Th1 effector-memory phenotype including IFN-γ and downregulated a panel of Treg-specific genes. Peripheral HY-specific green Tregs were adoptively transferred to Rag2−/− B6 male mice, to dissect the factors regulating ex-Treg differentiation. Development of ex-Tregs was more efficient in the mesenteric lymph node (mLN) than peripheral lymph node environment, correlating with a much greater level of IL-6 mRNA in mLN. In addition, the preferential development of ex-Tregs in mLN was significantly impaired by cotransfer of HY-specific naive CD4 T cells. Collectively, our study not only demonstrates the plasticity of Ag-specific Tregs in the context of the tumor environment, but also defines key molecular and cellular events that modulate ex-Treg differentiation. The Journal of Immunology, 2011, 186: 4557–4564.

The functional plasticity of peripheral Tregs is also evidenced by in vivo studies. We have recently demonstrated that a significant fraction of tumor Ag-specific green Tregs lost Foxp3 expression after adoptive transfer to tumor-bearing wild-type (WT) mice (11). Other groups have also shown that a large proportion of polyclonal green Tregs became GFP− and differentiated to Th1-like effector cells or follicular Th cells in Rag−/− (12, 13) and CD3−/− recipients (14), respectively. Furthermore, mice with attenuated expression of endogenous Foxp3 develop lymphoproliferative syndromes that are correlated with impaired suppressive function of Tregs and their conversion to effector cells (15). Moreover, using an elegant dual-reporter mouse model to distinguish T cells that constitutively express or have ceased to express Foxp3 in vivo, Bluestone and colleagues (16) found that a substantial fraction of peripheral Tregs lost Foxp3 and obtained an activated-memory phenotype even under steady-state conditions in unmanipulated healthy mice. Importantly, ex-Tregs that are present at a significantly higher frequency in autoimmune diabetes are autoreactive proinflammatory effector cells able to transfer diabetes (16).

Although the development of ex-Tregs has been studied in homeostatic (12–14) and autoimmune settings (15, 16), less information is available in terms of their differentiation in the context of tumor. In addition, adoptive transfer experiments generally use polyclonal Tregs, which has limited value in studying the role of Ag. More importantly, little is known regarding the cellular and molecular mechanisms of ex-Treg generation and regulation (17). In addition, whether the development of ex-Tregs is restricted to the CD25−/− subset of Tregs is controversial (13, 16). The plasticity of Foxp3 expression and the generation of ex-Tregs by thymic nTregs has also not been directly assessed.
To generate and characterize Ag-specific ex-Tregs, we modified a recently described in vivo model (11) by adoptively transferring HY-specific green Tregs (either thymic or peripheral) to MB49 tumor-bearing Rag2−/−B6 female recipients. Because of the preferential development of ex-Tregs in the lymphopenic environment (12–14), the use of Rag2−/−B6 female mice allows sufficient ex-Tregs to be isolated and characterized.

For exploring the cellular and molecular events that impact on ex-Treg development, a second in vivo model was applied in which green HY-specific peripheral Tregs were adoptively transferred to Rag2−/−B6 male mice in which HY is expressed ubiquitously.

Materials and Methods

**Mice**

Rag2−/−B6, Rag2−/−Thy1.2+ or Thy1.1+ Marilyn (18), Thy1.2+ Foxp3GFP B6 (19), and (Rag2−/− Marilyn × Foxp3GFP) F1 mice were described previously (8, 11, 20). All animal experiments were performed in accordance with Home Office Animals (Scientific Procedures) Act of 1986. In the absence of HY Ags, the development of Tregs in Rag2−/− Marilyn female mice (i.e., [Rag−/− Marilyn × Foxp3-GFP] F1) is likely to involve endogenous Vα TCR rearrangements allowing their positive selection on self MHC–peptide complexes (8, 11). In Rag2−/− Marilyn female mice without endogenous Vα TCR rearrangement, the frequency of Tregs is severely reduced. These results are shown in Supplemental Fig. 4. We have previously demonstrated that CD4+CD25+ cells from Rag2−/− Marilyn (8) or CD4+CD25+GFP+ cells from (Marilyn × Foxp3-GFP) F1 female mice [Rag−/−] (11) are functionally HY-specific Tregs both in vitro and in vivo.

**Tumor cell line**

In vitro culture of HY+ MB49 cells (21) was described elsewhere (11, 20).

**Purification of HY-specific naive CD4, green peripheral Treg, and green thymic nTregs**

This purification was conducted as described previously (8, 11, 20). Naive CD4 T cells (CD4+Vβ6+CD62L+CD44−) were FACSorted from spleen and LN cells of Rag2−/− Marilyn female mice. Green peripheral Tregs (GFP+CD4+Vβ6+CD25+) were FACSorted from spleen and pooled LN cells of (Rag2−/− Marilyn × Foxp3-GFP) F1 female mice. Green nTregs (GFP+CD4+CD8−CD25+) were FACSorted from CD8-depleted thymocytes of (Rag2−/− Marilyn × Foxp3-GFP) F1 female mice.

**Adoptive T cell transfer and tumor inoculation**

Adoptive T cell transfer was conducted by i.v. injection of various donor cell populations into Rag2−/−B6 female mice, which were either inoculated or not with MB49 cells (1 × 10⁶/mouse) by s.c. injection into the right flank on the same day. In some experiments, Rag2−/−B6 male mice were used as recipients.

**Analysis of representation of donor cell populations**

For MB49-bearing mice and their controls, analysis was conducted by staining inguinal LN (iLN), MB49-draining LN (dLN), spleen, or tumor mass (MB49) with anti-Vβ6 (or anti-CD4 PE, anti-CD8 FITC, and anti-CCR7 FITC), anti-Thy1.1 Alexa Fluor 647 (or CD4 PerCP), and anti-CD4 allophycocyanin (or anti-Thy1.2 allophycocyanin). The donor cells were identified before analyzing GFP, FR4, or CCR7 expression. For Rag2−/−B6 male mice, analysis was performed using peripheral LN (pLN), mesenteric LN (mLN), and spleen.

**Intracellular cytokine staining**

Intracellular cytokine staining was conducted as described previously (8, 11, 20).

**Reisolation of ex-Tregs and Tregs**

Ex-Tregs (GFP+CD4+Vβ6+) and unconverted Tregs (GFP−CD4+Vβ6+) were FACSorted from spleen and pooled LN cells of MB49-bearing Rag2−/−B6 female recipients of HY-specific green Tregs on day 12, and were subjected to either secondary transfer to Rag2−/−B6 male mice or quantitative real-time PCR (qRT-PCR) analysis. After FACSorting, a fraction of cells was subjected to intracellular anti-Foxp3 staining to confirm that ex-Tregs indeed do lose Foxp3.

**RNA and cDNA preparation**

RNA was extracted from sorted cells using an RNaseous-4PCR kit (Applied Biosystems, Warrington, U.K.), including DNase I digestion step. cDNA was made from 300 ng RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, U.K.). qRT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, U.K.), including DNase I digestion step, and the respective primers. The level of CCR7 expression was determined by qRT-PCR analysis. Results were presented as fold increase compared with Rag2−/−B6 female recipients of HY-specific green Tregs on day 12. MB49-draining LN (dLN) was defined as Rag2−/−B6 recipient mice. For MB49-bearing mice and their controls, analysis was conducted by staining inguinal LN (iLN), MB49-draining LN (dLN), spleen, or tumor mass (MB49) with anti-Vβ6 (or anti-CD4 PE, anti-CD8 FITC, and anti-CCR7 FITC), anti-Thy1.1 Alexa Fluor 647 (or CD4 PerCP), and anti-CD4 allophycocyanin (or anti-Thy1.2 allophycocyanin). The donor cells were identified before analyzing GFP, FR4, or CCR7 expression. For Rag2−/−B6 male mice, analysis was performed using peripheral LN (pLN), mesenteric LN (mLN), and spleen.

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**FIGURE 1.** Development of HY-specific ex-Tregs in vivo. A, Purity of HY-specific peripheral green Tregs. B and C, Analysis of ex-Tregs in vivo. HY-specific green Tregs (2 × 10⁶/mouse) were adoptively transferred to MB49-free (B) or MB49-bearing Rag2−/− B6 female mice (C) (4 mice/group). FACSorted analysis was conducted on day 12. Dot plots of one representative mouse of each group are shown. One representative experiment of three is shown. D–G, The percentage (D, E) and absolute number (F, G) of donor Tregs (D, F) and donor ex-Tregs (E, G) in iLN of control (black bars) and dLN of MB49-bearing mice (white bars) are shown. H, The purity of HY-specific thymic green nTregs. I, Analysis of ex-nTregs in vivo. HY-specific green nTregs (2 × 10⁶/mouse) were transferred to MB49-bearing Rag2−/− B6 female mice (n = 4). Analysis was conducted on day 12. FACSorted dot plots of one representative mouse of four are shown. One representative experiment of three is shown.
Transcription kit (Applied Biosystems), which facilitates quantitative conversion of mRNA to cDNA.

Quantitative real-time PCR and Global Pattern Recognition analysis

Mouse T Regulatory Phenotyping 383 StellARray quantitative PCR (qPCR) array plates (part #00188458; Lonza Sales AG, Basel, Switzerland) were used, with each plate containing 4 × 96 gene primer pairs. cDNA, Power Sybr green master mix (Applied Biosystems), and water were mixed, following the manufacturer’s directions, and 10 ml mix was added per well (1.5 ng cDNA). Plates were run on the 7900HT Real-Time PCR system (Applied Biosystems), under standard cycling conditions (50˚C for 2 min, 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min).

The resulting cycle threshold (Ct) data were analyzed using a modification of Global Pattern Recognition algorithm, GPR 2.0 (http://array.lonza.com/gpr/), comparing three replicates of GFP⁺ T Reg (control) with GFP⁻ T Reg (test) cDNA. When there is no difference between the Ct values of both groups, the genes are considered as “normalizers.” Each gene is compared with every normalizer in succession and the ∆Ct is calculated (ΔCt = Ct(Gene) – Ct(Normalize)). The ∆Ct for each group are then compared by a two-tailed, nonparametric Mann–Whitney U test or Student t test. Results are expressed as fold change, and those changes with a p value <0.05 are considered significant.

Statistical analysis

Data are presented as mean ± SEM. The comparison between groups was performed using a two-tailed, nonparametric Mann– Whitney U test or Student t test (p < 0.05 was considered as significant).

Results

Generation of HY-specific ex-Tregs in vivo

To develop an in vivo model by which ex-Tregs can be generated, we transferred highly purified, HY-specific, peripheral green Tregs (Fig. 1A) into MB49-free (Fig. 1B) or MB49-bearing Rag2⁻/⁻ B6 female mice (Fig. 1C). A significant accumulation of progeny of donor Tregs was found in tumor dLN, spleen, and tumor tissue on day 12 (Fig. 1C). However, the representation of donor Tregs in iLN and spleen in control mice was rather poor because of lack of Ag (Fig. 1B). Difference in the frequency (Fig. 1D) and absolute number (Fig. 1F) of donor Tregs between iLN and dLN was statistically significant.

Importantly, a fraction of the donor Tregs in dLN, spleen, and MB49 of tumor-bearing mice also became GFP⁺ (Fig. 1C), whereas the donor Tregs in the iLN and spleen of control mice remained 100% GFP⁻ (Fig. 1B). Tregs that have lost Foxp3 are henceforth referred to as ex-Tregs. Ex-Tregs in dLN have both statistically significant increased frequency (Fig. 1E) and absolute number (Fig. 1G), in comparison with those in iLN.

Peripheral Tregs isolated from the LN and spleen are composed of both nTregs and iTregs, whereas those from thymus are largely, if not exclusively, nTregs (4). FACS-sorted, HY-specific, thymic green Tregs (Fig. 1H) were transferred to MB49-bearing Rag2⁻/⁻ B6 female mice. Ex-Tregs were clearly present in dLN, spleen, and MB49 on day 12 (Fig. 1J); thus, nTregs can be converted to ex-Tregs after in vivo activation recapitulating the behavior of peripheral Tregs.

Development of ex-Tregs is not due to overexpansion by GFP⁻ contaminants

To rule out the possibility that the generation of ex-Tregs is caused by contaminating GFP⁻ conventional CD4 T cells, we designed a “contamination” experiment in which 1000 Thy1.1⁺ HY-specific naive CD4 T cells without or with 19,000 of Thy1.2⁺ HY-specific peripheral green Tregs were transferred to two groups of MB49-bearing Rag2⁻/⁻ B6 female mice.

In the absence of Tregs, a significant accumulation of progeny of donor naive CD4 T cells was found in dLN, spleen, and MB49 after 12 d, despite the limited number of input cells (Fig. 2A). However, in the presence of Tregs, representation of donor naive CD4 T cells was dramatically impaired with a 93–99% reduction in their accumulation in dLN, spleen, and MB49 (Fig. 2B). The statistical analysis of both frequency (Fig. 2C) and absolute number (Fig. 2D) of donor naive cells in dLN between these two groups also revealed significant differences. Because the naive CD4 T cells (5%) completely failed to “grow out” when cotransferred with excess Tregs (95%), the contribution of Foxp3⁻ contaminants must be negligible, and the emergence of ex-Tregs represents differentiation from donor Tregs. Using similar approaches, two groups have reached the same conclusions, although polyclonal T cell populations were used as donor cells (12, 13).

Ex-Tregs expand more potently than Tregs in Rag2⁻/⁻ B6 male mice

To compare the function of ex-Tregs and Tregs, we performed a secondary adoptive transfer. Tregs and ex-Tregs, generated as described in Fig. 1, were FACS sorted with >99% purity (Fig. 3A, 3B) and separately transferred to two groups of Rag2⁻/⁻ B6 male mice. Frequency of donor cells in recipients of ex-Tregs (Fig. 3D) was much greater than that in recipients of Tregs on day 28 (Fig. 3C). These differences were statistically significant (Fig. 3E–G). In addition, the progeny of donor ex-Tregs showed a higher frequency in mLN but a lower frequency in pLN (Fig. 3D). Interestingly, a significant fraction of donor ex-Tregs in spleen but
Not in mL or pLN re-expressed GFP (Fig. 3H). These differences were also statistically significant (Fig. 3I).

Ex-Tregs downregulated Treg-specific genes but upregulated genes characteristic of a Th1 effector-memory phenotype

Tregs and ex-Tregs, generated as described in Fig. 1, were FACS sorted before comparing gene expression profiles. As expected, Foxp3 expression was reduced by 10-fold in ex-Tregs, which also showed a 116-fold decrease of Gpr83 (G protein-coupled receptor 83), a Treg-specific surface marker (22, 23). Other Treg-associated genes including Ctit4 (CTLA4) (24), Folr4 (FR4) (25), Ntse (CD73) (26), and Ccr7 (CCR7) (27) were also downregulated (Fig. 4A). In contrast, ex-Tregs displayed a significant increase of Ifng (IFN-γ), Cd80 (CD80), Gzmb (granzyme B), and Gzma (granzyme A) (Fig. 4B). To a lesser extent, Th1 cytokine genes including Csf2 (GM-CSF), Tnf (TNF-α), and Il2 (IL-2) were also moderately but significantly upregulated in ex-Tregs. Interestingly, several Treg-related genes including Runxl (28, 29) and Gal1 (galectin 1) (30) were maintained in ex-Tregs, indicating that re-differentiation was incomplete.

Validation of qRT-PCR data by FACS analysis

To confirm that ex-Tregs express more IFN-γ than Tregs with sustained Foxp3 at the protein level, we used intracellular staining. MB49-bearing Rag2−/− B6 female recipients of HY-specific Tregs were prepared as described in Fig. 1. On day 12, dLN cells were subjected to a standard intracellular IFN-γ procedure after a brief in vitro restimulation (Fig. 5A). The frequency of IFN-γ–producing cells in ex-Tregs was significantly higher than that in Tregs. The mean fluorescence intensity (MFI) of IFN-γ in ex-Tregs was also higher than that in Tregs; thus, overall ex-Tregs produced more IFN-γ than Tregs. Neither IL-4 nor IL-17 was detectable in the same experiment (Supplemental Fig. 1), indicating that Tregs differentiated into Th1 but not Th2 or Th17 cells. We also confirmed that expression of both FR4 and CCR7 was significantly reduced on ex-Tregs (Fig. 5B).

MB49 tumor mass is an inflamed tissue that favors the development of ex-Tregs

For the induction of ex-Tregs in vivo, lymphopenic mice are more efficient than lymphoreplete mice (12–14). Nevertheless, we
previously reported that ex-Tregs were also present in MB49-bearing WT B6 recipients of HY-specific green Tregs (11). Interestingly, the frequency of ex-Tregs in tumor tissues is higher than that in dLN isolated from the same mice (11), indicating that the tumor provides an environment that favors ex-Treg development. When gene expression profiles of cytokines/chemokines/receptors were compared between MB49 tissue and MB49 cell line, we found that 22 genes were upregulated in tumor tissue with only one gene being downregulated (IL-13, 17-fold decrease) (Fig. 6). IL-1β, a well-known proinflammatory cytokine, showed a 428-fold increase. The highest fold increase in expression was seen for Cxcl9, which showed a 2600-fold increase, followed by Ccl3, Ccl4, Ccl12, and Ccl5. Increased expression of a panel of chemokine receptors including CXCR3, the receptor for CXCL9, was also observed in MB49 tissue. Taken together, these qRT-PCR data suggested that MB49 tissue provides a highly inflammatory environment.

HY-specific green Tregs preferentially differentiate into ex-Tregs in mesenteric but not pLN of Rag2<sup>−/−</sup> B6 male recipients

We also studied the differentiation of ex-Tregs in the second model in which HY-specific green peripheral Tregs were adoptively transferred into Rag2<sup>−/−</sup> B6 male mice. Strikingly, the complete conversion of Tregs into ex-Tregs was seen in mLN but not in pLN or spleen on day 14 (Fig. 7A, 7B). When mLN cells were compared with pLN for cytokine/chemokine/receptor gene expression, seven genes showed significant upregulation in mLN (Fig. 7C), of which IL-6 mRNA displayed a >300-fold increase. A moderate increase of IL-1R antagonist, CXCL10, IL-10, CXCR4, IL-21, and IL-2 was also observed.

Regulation of ex-Treg differentiation by naive CD4 T cells

To explore whether HY-specific naive CD4 T cells could influence HY-specific ex-Treg development in Rag2<sup>−/−</sup> B6 male recipients, we performed a cotransfer experiment. As shown in Fig. 7D and 7E, the copresence of naive CD4 T cells (1:1 ratio) led to a remarkable reduction (up to 80%) in the frequency of ex-Tregs in mLN. A modest but significant decrease (40%) of ex-Treg frequency was also seen in pLN but not in spleen. Thus, feedback
from naive CD4 T cells acts to maintain the expression of Foxp3 by Tregs. In the tumor model, the development of ex-Tregs in MB49 tissues was also significantly impaired when naive CD4 T cells coexisted (Supplemental Fig. 2).

**Discussion**

In this study, we have exclusively used Rag<sup>−/−</sup> B6 mice as recipients of HY-specific green Tregs from B6.Foxp3<sup>gfp</sup>.Marilyn TCR-transgenic mice. This is in contrast with the study by Rubtsov et al. (31), in which lymphoreplete NOD mice were used as recipients of pancreatic-islet-Ag–specific GFP<sup>+</sup> Tregs from NOD. Foxp3<sup>gfp</sup>.BDC2.5 TCR-transgenic mice. The different conclusions in terms of Foxp3 stability are likely to be due to the use of these distinct types of recipient mice. In addition, the level of inflammation present in nonmalignant tissue (prediabetic pancreas of 12-wk-old NOD mice) and malignant tissue might be dramatically different.

We have examined the expression of intracellular Foxp3 by ex-Tregs (FACS sorted from MB49-bearing mice based on the loss of GFP expression), before the secondary transfer (as shown in Fig. 3) and qRT-PCR analysis (as shown in Fig. 4). These GFP<sup>−</sup> ex-Tregs had lost Foxp3 expression since staining with anti-Foxp3 mAb was also negative, whereas positive Foxp3 staining was seen in the sorted GFP<sup>+</sup> population. These results are shown in Supplemental Fig. 3. Therefore, we can confirm that Tregs that lost GFP are indeed Foxp3<sup>+</sup>, which appears to be consistent with the RT-PCR analysis showing that there is a significant downregulation (10-fold) of Foxp3 mRNA expression in ex-Tregs compared with that in unconverted green Tregs (Fig. 4A).

In the first part of this study, we characterized HY-specific ex-Tregs (Figs. 3–5, Supplemental Fig. 1), which have developed in an in vivo tumor model (Fig. 1), and excluded the possibility that they represent the outgrowth of contaminating conventional CD4 cells (Fig. 2). We also identified several unique features of HY-specific ex-Tregs regarding their origin, differentiation, and phenotype.

First, ex-Tregs differentiate from CD25<sup>+</sup> peripheral Tregs (Fig. 1C). This is consistent with one report showing that a fraction of islet autoantigen-specific CD4<sup>+</sup>GITR<sup>+</sup>GFp<sup>+</sup> cells (of which most are CD25<sup>+</sup>) lose Foxp3 after transfer to TCR<sup>−/−</sup> NOD mice (16), but appears to conflict with another study suggesting that the plasticity of Tregs is exclusive to the CD25<sup>+</sup>-Treg subset (13). Failure to detect ex-Tregs in Rag<sup>−/−</sup> B6 recipients of CD25<sup>+</sup> Tregs by Komatsu et al. (13) could be because: 1) the test was carried out only at an early time point (day 5); and 2) presumably only pLN (whose environment is less inflammatory than that of mLN) was analyzed. Indeed, when analysis was conducted at a later time point (day 28), 90% of donor CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs (of which most are CD25<sup>+</sup>) became Foxp3<sup>+</sup> recovered from intraepithelial lymphocytes (12) and Peyer’s patches (14). It appears that an inflammatory milieu can promote ex-Treg commitment (12, 14, 16). Given that IL-2 is critical to maintain stable expression of Foxp3 in Tregs (32), it has been suggested that the CD25<sup>−</sup> Treg subset might be more susceptible to Foxp3 loss (13). But it does not necessarily indicate that the CD25<sup>−</sup> Treg subset is the sole population from which ex-Tregs are generated. This is because on homeostatic expansion in lymphopenic mice, the CD25<sup>−</sup> Tregs lose CD25 (33), and vice versa, CD25<sup>−</sup> Tregs also regain CD25 expression (34).

Second, nTregs (i.e., thymic green Tregs) are shown to be able to differentiate to ex-Tregs (Fig. 1H, 1I). Previous studies exclusively used peripheral Tregs (10, 12–14, 16), containing both nTregs and iTregs, which differentially express Helios (4). As a transcription factor, Helios has a limited value in separating the two subsets present in peripheral Tregs. The use of thymic green Tregs as an alternative strategy provides direct evidence that in vivo, nTregs can be the precursors of ex-Tregs.

Third, ex-Tregs have characteristics of Th1 rather than Th17 or Th2 cells (Fig. 5A, Supplemental Fig. 1). These data are compatible with a study by Zhou et al. (16) showing that polyclonal ex-Tregs in LN produced IFN-γ but not IL17, whereas those in Peyer’s patches made both. Therefore, the cytokine profiles of ex-Tregs also depend on the location and environment where they are generated.

Finally, ex-Treg population is heterogeneous. qPCR analysis revealed that expression of some Treg-related genes, including Runx1 (28, 29) and galectin 1 (30), is maintained in ex-Tregs (Fig. 4). On their secondary transfer to Rag2<sup>−/−</sup> B6 male mice, some ex-Tregs in spleen but not LN became Foxp3<sup>+</sup> (Fig. 3H), suggesting that re-expression of Foxp3 also depends on the tissue environment. Foxp3 re-expression by polyclonal ex-Tregs has been reported (13), although it is unclear whether ex-Tregs in spleen are more competent than those in LN at regaining Foxp3.

Treg conversion in lymphocyte-sufficient hosts appears to be much less efficient than that in lymphocyte-deficient hosts, according to our own study (11) and others (12, 13, 16), which raises concerns over the biological significance of the conversion of Tregs in vivo. Nevertheless, Tregs can be converted to ex-Treg under nonlymphopenic conditions. Interestingly, this conversion takes place in inflamed tissue-expressing Ags, including the MB49 tumor mass (11) and the pancreas of prediabetic NOD mice (16). From these studies, it has been proposed that the conversion of Tregs under physiological conditions would contribute to the development of autoimmune diseases and cancer surveillance; in addition, the generation of effector-like ex-Tregs at sites of infected tissue might help initiate clearance viral infection (17). Exploration of the biological relevance of Treg conversion in WT mice needs robust model systems and novel approaches. Commonly used strategies are to perform the secondary transfer of ex-Tregs to lymphopenic hosts, as shown by us in this article and by others (13, 14, 16). To directly examine ex-Treg function in recipient mice, we will be using a Treg depletion system (green Treg coexpressing Foxp3/GFP/DTR fusion protein).
The second part of this study explored the cellular events controlling the development of ex-Tregs. There are two key observations. The first is that the microenvironment of mLNs but not pLN favors the generation of ex-Tregs (Fig. 7A, 7B). A subsequent comparison of gene expression profiles revealed that IL6 mRNA shows a remarkable increase (>300-fold) in mLNs compared with pLN cells (Fig. 7C). IL-6 alone can downregulate Foxp3 expression by TCR-stimulated Tregs, which can be further promoted by the copresence of IL-1 and IL-21 (9, 10, 14). Moreover, IL-6 induces remethylation of the Foxp3 nonintronic upstream CpG-rich island and closes the chromatin structure of the Foxp3 locus (35). Although the link between increased IL-6 expression and efficient ex-Treg differentiation in mLNs needs to be confirmed by in vivo studies, higher IL-6 expression by mLNs provides a genetic basis for greater development of ex-Tregs in this particular tissue, which significantly extends previous studies showing that inflammatory environments such as gut-associated tissues (12, 14) and islet of NOD mice (16, 17) can facilitate the development of ex-Tregs.

It is of interest to test whether ex-Tregs that resulted from in vivo conversion would have pathological consequences such as the development of colitis. However, there is a concern that the activities of ex-Tregs would be limited by the coexistence of nonconverted Tregs, especially in pLN where the Treg conversion was incomplete. Another concern is that the use of HY-specific Tregs is the main focus in the current investigation, whereas the development of colitis in B6 mice appears to require a polyclonal CD4+ T cell response. To explore whether ex-Tregs can induce a pathological response in vivo, unconverted Tregs would need to be removed as described earlier. The effector potential of ex-Tregs could be assessed by their anti-MB49 immunity or the development of graft-versus-host disease-like disease after transfer to irradiated male recipients, which we have seen after adoptive transfer of conventional Marilyn CD4+ T cells (J.-G. Chai, unpublished observations). It is also of interest to use Rag2−/− IL6 KO mice to examine whether these hosts fail to convert the Tregs.

The second observation is that HY-specific naive CD4+ T cells can partially prevent ex-Treg development in vivo (Fig. 7D, Supplemental Fig. 2). Similar results have been reported by others using polyclonal Tregs (12, 36). Although the precise molecular mechanisms for regulating Foxp3 expression in Tregs by cotransferred naive CD4 cells are still elusive, involvement of cytokines including IL-2 and TGF-β have been suggested. Duarte et al. showed that lymphopenia-induced loss of Foxp3 by polyclonal Tregs was accelerated by anti–IL-2 treatment but prevented by IL-2 administration (12). Similarly, IL-2 treatment can restore CD25 expression and Treg function in inflamed islet of NOD mice (37). TGF-β produced by Tregs is required for maintaining Foxp3 expression and protection from apoptosis (38).

What are the implications of this study? First, some features of Tregs revealed by previous studies might be attributable to alternative interpretations because of the instability of Foxp3 expression. For example, Tregs act as effector cells in lymphopenic hosts (39); Tregs secrete granzyme B and/or perforin to kill DC, CD8,
and NK cells in tumor-ILN (40) or in tumor tissues (41); and Tregs preferentially expand in response to cancer vaccines (42). Second, there is concern over the use of Treg adoptive therapy for autoimmune diseases (16, 17). Because IL-6 plays a key role in converting Tregs to ex-Tregs (9, 10), the efficiency of adoptive Treg therapy may be enhanced by blockade of IL-6 signaling pathways. Finally, the promotion of ex-Treg development in the context of tumors can be of therapeutic benefit.

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Disclosures
The authors have no financial conflicts of interest.

References
**Figure S1. Ex-Tregs produce IFNγ but not IL4 or IL17.**

This was conducted as described in Fig.5A, except that two additional anti-IL4<sup>PE</sup> and anti-IL17<sup>PE</sup> were also utilized for intracellular staining.
Figure S2. Development of ex-Tregs in MB49-bearing mice was impaired by cotransferred naïve CD4 T cells.

A and B. HY-specific green Treg (Thy1.2) (1x10⁴/mouse) alone (A) or mixed with an equal number of HY-specific naive CD4 T cells (Thy1.1) (B) were transferred to two groups of MB49-bearing Rag2⁻/⁻ B6 females (n=4). Analysis was conducted on day 12 by staining of dLN, MB49, or SP cells with anti-CD4<sub>PE</sub>, anti-Thy1.1<sub>PerCP</sub> and anti-Thy1.2<sub>APC</sub>. Donor Treg cells identified as CD4⁺Thy1.2⁺ were analysed for Foxp3. FACS profiles of one representative mouse of four are shown. One representative experiment of three is shown.

C. Comparison of the frequency of ex-Treg cells in the absence (black bars) or presence of naïve CD4 T cells (white bars).
**Figure S3. Ex-Treg cells are Foxp3 negative.**

The development, identification and re-isolation of HY-specific ex-Treg and unconverted Treg cells were described in Figure 1a-c and Figure 3a-b. After FACS sorting, a fraction of ex-Treg (CD4+GFP) (left hand panel) and unconverted green Treg cells (right hand panel) were subsequently subjected to intracellular Foxp3 staining using anti-Foxp3^FITC mAb (e-Biosciences).
Figure S4. Comparison of green Treg cells frequency between Rag\(^{+/-}\) and Rag\(^{-/-}\) Marilyn females co-expressing Foxp3/GFP.

The PBL from Marilyn females co-expressing Foxp3/GFP as a fusion protein on Rag-sufficient- (Rag\(^{+/-}\)Marilyn, n=>40) or Rag-deficient background (Rag\(^{-/-}\)Marilyn, n=8) were stained with anti-V\(\beta\)\(^6\)PE, anti-CD4\(^{\text{PerCP}}\) and anti-B220\(^{\text{APC}}\). The frequency of Treg cells is represented by the percentage of GFP\(^{+}\) cells within gated CD4\(^{+}\)V\(\beta\)\(^6^{+}\) population. Absence of both B220\(^{+}\) and CD4\(^{+}\) cells indicates the Rag-deficiency and presence of a large fraction of CD4\(^{+}\)V\(\beta\)\(^6^{+}\) suggests a phenotype being TCR-transgenics. **, p<0.01.