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

Caroline Addey,*1 Matthew White,*1 Lang Dou,*1 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.

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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+CD62LhiCD44lo) were FACS sorted from spleen and LN cells of Rag2−/− Marilyn female mice. Green peripheral Tregs (GFP+CD4+Vβ6+CD25+) were FACS sorted from spleen and pooled LN cells of (Rag2−/− Marilyn × Foxp3-GFP) F1 female mice. Green nTregs (GFP+CD4+CD8−CD25+) were sorted 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-Vβ6PE (or anti-CD4PE, anti-FR4PE, and anti-CCR7PE), anti-Thy1.1PECF (or CD4PEB72), and anti-CD4allophycoerythrin (or anti-Thy1.2allophycoerythrin). 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 FACS sorted 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 FACS, 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 RNAqueous-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
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.lonz.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 ΔCt is calculated (ΔCt Gene = Ct Gene − Ct Normalizer). The ΔCt for each group are then compared by a two-tailed unpaired Student t test. Results are expressed as fold change, and those changes with a p value <0.05 are considered significant.

Statistic 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 become 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.
not in mLN or pLN re-expressed GFP (Fig. 3 H). These differences were also statistically significant (Fig. 3 I).

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 Ctla4 (CTLA4) (24), Folr4 (FR4) (25), Nt5e (CD73) (26), and Ccr7 (CCR7) (27) were also downregulated (Fig. 4 A). In contrast, ex-Tregs displayed a significant increase of Ifng (IFN-γ), Cd80 (CD80), Gzmb (granzyme B), and Gzma (granzyme A) (Fig. 4 B). 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 Runx1 (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. 5 A). 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. 5 B).

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 peripheral 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 \textit{Ccl3}, \textit{Ccl4}, \textit{Ccl12}, and \textit{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\(^{-/-}\) 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\(^{-/-}\) 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\(^{-/-}\) 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 remarked 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
was also negative, whereas positive Foxp3 staining was seen in the different conclusions had lost Foxp3 expression since staining with anti-Foxp3 mAb two genes were upregulated in tumor tissue with only one gene being downregulated (IL-13, 17-fold decrease). These experiments were repeated twice and similar results were obtained.

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\(^{-/-}\) B6 mice as recipients of HY-specific green Tregs from B6.Foxp3\(^{gfp}\). 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\(^+\) Tregs from NOD. Foxp3\(^{gfp}\).BCD2.5 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\(^+\) 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\(^+\) population. These results are shown in Supplemental Fig. 3. Therefore, we can confirm that Tregs that lost GFP are indeed Foxp3\(^-\), 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\(^+\) peripheral Tregs (Fig. 1C). This is consistent with one report showing that a fraction of islet autoantigen-specific CD4\(^+\)GITR\(^{hi}\)GFP\(^+\) cells (of which most are CD25\(^+\)) lose Foxp3 after transfer to TCR\(^{-/-}\) NOD mice (16), but appears to conflict with another study suggesting that the plasticity of Tregs is exclusive to the CD25\(^-\)-Treg subset (13). Failure to detect ex-Tregs in Rag\(^{-/-}\) B6 recipients of CD25\(^+\) 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\(^+\)Foxp3\(^+\) Tregs (of which most are CD25\(^+\)) became Foxp3\(^-\) 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\(^-\) Treg subset might be more susceptible to Foxp3 loss (13). But it does not necessarily indicate that the CD25\(^-\) Treg subset is the sole population from which ex-Tregs are generated. This is because on homeostatic expansion in lymphopenic mice, the CD25\(^-\) Tregs lose CD25 (33), and vice versa, CD25\(^+\) 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-\(\gamma\) 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\(^{-/-}\) B6 male mice, some ex-Tregs in spleen but not LN became Foxp3\(^+\) (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).
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FIGURE 7. Microenvironment of mLN in Rag2\(^{-/-}\) B6 male mice favors ex-Treg development. A, HY-specific green Tregs (Thy1.1)(1 \(\times\) 10^7/mouse) were transferred to Rag2\(^{-/-}\) B6 male mice (\(n = 4\)). Analysis was conducted on day 14 by staining of pLN, mLN, or spleen cells with anti-V60^Het, anti-Thy1.1^PerCP, and anti-CD4^Allophycocyanin. Donor Tregs identified as V60^HetThy1.1^ were analyzed for Foxp3 expression. FACS, Comparison of gene expression profiles between pLN favors ex-Treg development. B, Inhibition of ex-Treg differentiation by cotransferred naive CD4 cells. HY-specific green Tregs (Thy1.1)(1 \(\times\) 10^7/mouse) mixed with an equal number of HY-specific naive CD4 T cells (Thy1.2) were transferred to Rag2\(^{-/-}\) B6 male mice (\(n = 4\)). Analysis was conducted on day 14, as described in Fig. 6A. FACS profiles of one representative mouse of four are shown. One representative experiment of three is shown. C, Comparison of the frequency of ex-Tregs in various tissues. C, Comparison of gene expression profiles between pLN and mLN, pLN and mLN cells from a Rag2\(^{-/-}\) B6 male mouse were subjected to RNA extraction and cDNA conversion before running qPCR array assay using mouse cytokine/chemokine/receptor 383 StellARray plates. One representative experiment of two is shown. D, Inhibition of ex-Treg differentiation by cotransferred naive CD4 cells. HY-specific green Tregs (Thy1.1)(1 \(\times\) 10^7/mouse) mixed with an equal number of HY-specific naive CD4 T cells (Thy1.2) were transferred to Rag2\(^{-/-}\) B6 male mice (\(n = 4\)). Analysis was conducted on day 14, as described in Fig. 6A. FACS profiles of one representative mouse of four are shown. One representative experiment of three is shown. E, Comparison of the frequency of ex-Tregs in the absence (black bars) or presence of cotransferred naive CD4 T cells (white bars).
and NK cells in tumor-dLN (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
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