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TCR Signaling Events Are Required for Maintaining CD4 Regulatory T Cell Numbers and Suppressive Capacities in the Periphery

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CD4 regulatory T cells (Tregs) can be subdivided into two subsets according to Ly-6C expression in the periphery. Phenotypic analysis, imaging, and adoptive-transfer experiments of peripheral Ly-6C− and Ly-6C+ Tregs reveal that the nonexpression of Ly-6C by ∼70% of peripheral Tregs depends on TCR signaling events. Interestingly, Ly-6C− Tregs express higher surface amounts of key immunosuppressive molecules than do Ly-6C+ Tregs and produce constitutively anti-inflammatory cytokines. In line with their phenotype, Ly-6C+ Tregs exhibit poor suppressive capacities in vitro and in vivo. Finally, although Ly-6C− Tregs maintain their numbers with age, Ly-6C+ Tregs gradually disappear. Altogether, our data strongly suggest that both the survival and suppressive functions of peripheral CD4 Tregs rely on their ability to receive strong TCR signals. The Journal of Immunology, 2014, 193: 5914–5923.

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oxp3-expressing CD4 regulatory T cells (Tregs) are critically involved in the maintenance of self-tolerance and regulation of immune responses (1, 2). In particular, they are key players in the prevention of various autoimmune and inflammatory disorders. CD4 Tregs can be divided into two classes, depending on their ontogeny: natural Tregs (nTregs), which arise in the thymus, and induced Tregs (iTregs), which are generated in the periphery. Studies in mice suggest that the TCR has an instructive role in inducing commitment of developing thymocytes into the Treg lineage (3, 4). This model is supported by the observation of a limited amount of overlap (10–20%) between the TCR sequences expressed within the CD4 conventional T cell (Tconv) repertoire and the CD4 Treg repertoire (5, 6). Interestingly, overlapping is more important when the Treg repertoire is compared with the repertoire of pathogenic autoreactive effector T cells (7, 8). Altogether, these data suggest that nTregs develop in the thymus as a result of strong TCR signals, thereby yielding a compartment that is enriched for reactivity against self-Ags.

A second pathway for Treg generation resides in the conversion of Tconv into iTregs within the periphery (9). Commitment to the iTreg lineage is considered to depend mostly on the immunological context in which Tconvs are imersed at the time of their activation. In particular, exposure to TGF-β (in the absence of IL-6) appears to be critical for iTreg generation. Nevertheless, differentiation of Tconvs into iTregs upon stimulation also relies on intrinsic factors, such as the avidity of the TCR that they express for both self (10) and foreign ligands (11). Indeed, Gottschalk et al. (11) showed that a low dose of a strong agonist resulted in maximal induction of iTregs. Moreover, we recently showed that the ability of a naïve CD4 T cell to commit into the iTreg lineage pathway upon appropriate stimulation is shaped by its capacity to interact with self-Ags in its original environment (10).

Thus, the Treg repertoire of both nTregs and iTregs is enriched in cells expressing high-affinity TCRs for self-Ags. We recently found that Ly-6C expression allows the discrimination of low and high self-reactive naïve CD4 T cells (10). In this article, we show that peripheral CD4 Tregs also can be subdivided into two subsets according to Ly-6C expression, and we describe the ontogeny, homeostasis, and functional abilities of these newly defined CD4 Treg subsets.

Materials and Methods

Mice

C57BL/6 mice (CD45.2) were obtained from Charles River Laboratories. C57BL/6 CD45.1 mice and C57BL/6 CD3−/− mice were maintained in our own animal facilities, under specific pathogen–free (SPF) conditions. C57BL/6 Foxp3-GFP CD45.2 mice were initially obtained from Dr. Bernard Malissen (Centre d’Immunologie de Marseille-Luminy, Marseille, France) (12). C57BL/6 Foxp3-GFP CD45.2 mice were crossed with C57BL/6 CD45.1 mice to generate C57BL/6 Foxp3-GFP CD45.1 mice. C57BL/6 Foxp3-GFP CD45.1 mice were crossed with C57BL/6 MHC II−/− mice (13) to obtain C57BL/6 Foxp3-GFP MHC II−/− CD45.1 mice. C57BL/6 germ-free (GF) mice and the related control C57BL/6 GF mice were obtained from CDTA Orléans. Donor and recipient mice were sex matched. Four- to six-week-old mice were used for experiments unless otherwise indicated. Experiments were carried out in accordance with the guidelines of the French Veterinary Department. All procedures performed were approved by the Paris-Descartes Ethical Committee for Animal Experimentation (decision CEEA34-002.12).

Cell suspensions

Peripheral lymph nodes (pLNs), mesenteric LNs (mLNs), cervical LNs (cLNs), Peyers’ patches (PPs), spleen, and thymus were homogenized and passed through a nylon cell strainer (BD Falcon) in RPMI 1640 GlutaMAX (Life Technologies) supplemented with 10% FCS (Biochrom) for adoptive transfer and cell culture (LNs only) or in 5% FCS, 0.1% NaN3 (Sigma-Aldrich) in PBS for flow cytometry.
Tissue processing

Ketamine-xylazine–anesthetized mice were perfused intracardially with cold PBS. Small intestines were opened longitudinally after removal of PPs, washed in successive HBSS 5% FCS baths, and incubated in RPMI 1640 20% FCS with collagenase D (1 mg/ml) and DNase I (0.1 mg/ml, both from Roche) for 30 min at 37°C. Cells were then recovered after Percoll gradient separation (40–70%).

Fluorescence staining and flow cytometry

Cell suspensions were collected and dispensed into 96-well round-bottom microtiter plates (Greiner Bioscience; 6 × 10^5 cells/well). Surface staining was performed as described (14, 15). Briefly, cells were incubated on ice, for 15 min/step, with Abs in 5% FCS (Biochrom), 0.1% NaN3 (Sigma-Aldrich) PBS. Each cell staining reaction was preceded by a 15-min incubation with a purified anti-mouse BD6/32 Ab (PhytoPE/III block; 2.4G2). For determination of intracellular cytokine production, cells were stimulated with 0.5 μg/ml PMA, 0.5 μg/ml ionomycin, and 10 μg/ml brefeldin A (all from Sigma-Aldrich) for 2 h at 37°C. Cells were then stained for surface markers, fixed in 2% paraformaldehyde in PBS, and permeabilized with 0.5% saponin, followed by labeling with specific cytokine Abs. The Foxp3 Staining Buffer Set (eBioscience) was used for Foxp3, CTLA-4, and Ki-67 intracellular staining. For determination of caspase-3 activity, cells were resuspended in 200 μl Fluorescence-activated cell sorting (FACS) buffer and stained with H&E and then analyzed in a blinded fashion. Each segment was graded for scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with mild to moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; grade 3, moderate inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; and grade 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion, and loss of intestinal glands.

Blocking T cell entry into LNs

C57BL/6 Foxp3-GFP mice were injected i.p. or not with 200 μg anti-α-β (clone M17/4) and anti-ε4 (clone PS/2) Abs (Bio X Cell) to block LN entry. Twenty-four hours later, cLNs, mLNs, and spleen were harvested, and CD4 T cells were analyzed.

Immunoblot analysis

Ly-6C− and Ly-6C+ CD4 Tregs (3 × 10^5 cells/condition) were sorted by flow cytometry, as described above, washed in ice-cold PBS, and lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 8], 5 mM Na3P04, 1 mM Na2VO4, 20 mM NaPO4 [pH 7.6], 3 mM 1,2-bis(glycerolphosphate), 10 mM NaF, 1% Triton X-100, complete protease, and phosphatase inhibitor mixture [Roche]) for 15 min on ice. Samples were centrifuged, and whole-cell lysates were harvested. Unreduced proteins were separated on a 12% SDS-PAGE, transferred on a nitrocellulose membrane, and immunoblotted first with an anti-phosphotyrosine Ab (clone 4G10; Millipore) and, after stripping, with an anti-CD3ζ Ab (clone 6B10.2; Santa Cruz Biotechnology). Signals were quantified using ImageJ software. The ratio p/total was estimated as previously described (17, 18).

Immunofluorescence microscopy

Ly-6C− and Ly-6C+ CD4 Tregs were sorted by flow cytometry as GFP+ cells using a FACSARia III flow cytometer (BD Biosciences). List-mode data files were analyzed using Diva software (BD Biosciences). Data acquisition and cell sorting were performed in the Cochin Immunobiology facility.

Adoptive transfer of CD4 Tregs

CD4 T cells were purified from LNs (pooled superficial cervical, axillary, brachial, inguinal, and mesenteric LNs) or thymi of C57BL/6 Foxp3-GFP mice by incubating cell suspensions on ice for 20 min with a mixture of anti-CD8α (53-6.7), anti-CD11b (Mac-1), and anti-CD19 (1D3) Abs obtained from hybridoma supernatants and then with magnetic beads coupled to anti-rat IgG (Dynal Biotech). Then, purified CD4 T cells were labeled with biotinylated anti-ly-6C (AL21; BD Biosciences) and Pacific Blue-conjugated streptavidin (Invitrogen). Ly-6C− and Ly-6C+ CD4 Tregs were sorted by flow cytometry as GFP+ cells using a FACSAria III flow cytometer (BD Biosciences) and injected i.v. into sex-matched recipient mice.

In vitro suppression assay

Purified Ly-6C− or Ly-6C+ CD4 Tregs were sorted by flow cytometry, and the suppressive capacities of these highly purified Foxp3-expressing cells were assessed, as previously described (16). Briefly, CD4 T cells (GFP+ CD4+ T cells) were purified from LNs of C57BL/6 Foxp3-GFP mice, labeled with anti-CD8α (clone M17/4) and anti-α-β (clone 53-6.7); and anti-CD11b (clone M17/4) and anti-CD19 (clone 1D3); and anti-CD19 (clone 1D3) Abs obtained from hybridoma supernatants and then with magnetic beads coupled to anti-rat IgG (Dynal Biotech). Then, purified CD4 T cells were labeled with biotinylated anti-ly-6C (AL21; BD Biosciences) and Pacific Blue-conjugated streptavidin (Invitrogen). Ly-6C− and Ly-6C+ CD4 Tregs were sorted by flow cytometry as GFP+ cells using a FACSARia III flow cytometer (BD Biosciences) and injected i.v. into sex-matched recipient mice.

In vitro suppression assay

CD4 Tregs were purified from C57BL/6 Foxp3-GFP mice, as described above. Naive CD4 T cells were purified from C57BL/6 Foxp3-GFP mice, as described previously (10). A total of 5 × 10^5 naive CD4 T cells was injected i.v. into sex-matched recipient mice, alone or together with 5 × 10^5 Ly-6C− or Ly-6C+ CD4 Tregs. The weight of recipient mice was monitored every 2 d, and mice were sacrificed either 7 wk after transfer or when they reached 80% of their initial weight.

Colitis scoring

For the colitis model, colons were removed and fixed in PBS containing 10% formaldehyde. Five-micrometer paraffin-embedded sections were cut and stained with H&E and then analyzed in a blinded fashion. Each segment was given a score of 0 to 4: grade 0, no significant changes; grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with mild to moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; grade 3, moderate inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; and grade 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion, and loss of intestinal glands.

Quantitative RT-PCR analysis

Total RNA was isolated from flow-cytometry–sorted cells, as described above, and reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen) using 100 ng Random Hexamers. Quantitative PCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and a real-time PCR system (ABI7300; Applied Biosystems), according to standard PCR conditions. For quantitative calculations, results were normalized to hprt expression. Primers used are described in Supplemental Table I.

Statistics

Data are expressed as mean ± SEM, and the significance of differences between two series of results was assessed using the Student unpaired or paired t test. The p values < 0.05 were considered significant.

Results

Peripheral CD4 T cells can be subdivided into two subsets on the basis of Ly-6C expression

Throughout this study, CD4 T cells were defined as Foxp3+ CD4+ CD8α− CD3+ cells (Supplemental Fig. 1). CD4 T cells from sec-
Secondary lymphoid organs of C57BL/6 mice can be subdivided into two subsets according to Ly-6C expression (Fig. 1A). In all secondary lymphoid organs, ~30% of CD4 Tregs expressed Ly-6C. In contrast, the proportion of Ly-6C+ CD4 Tregs was substantially decreased in the thymus. Therefore, as for naive CD4 T cells, Ly-6C expression divides the CD4 Treg compartment into two subsets in secondary lymphoid organs.

We then injected Ly-6C− CD4 Treg thymocytes into mice expressing or lacking MHC class II molecules (Fig. 1B). Ly-6C− CD4 Treg thymocytes gave rise to both Ly-6C− and Ly-6C+ CD4 Tregs after transfer into MHC class II–competent recipient mice (Fig. 1C). Thus, nTregs are comprised within both Ly-6C− and Ly-6C+ cells. Interestingly, the majority of CD45.2+ CD4 Tregs recovered from MHC class II–deficient recipient mice 7 d after transfer expressed Ly-6C, and nearly all of them expressed high amounts of this marker 2 and 4 wk after transfer. This result suggests that, as for naive CD4 T cells (10), downmodulation of Ly-6C expression by CD4 Tregs depends on continuous interactions with MHC class II molecules.

It was shown recently that neuropilin-1 (Nrp-1) expression distinguishes nTregs from iTregs in the periphery. More precisely, unlike nTregs, iTregs would not express this marker (19, 20). As shown by other investigators (19), a significant proportion of Nrp-1− cells among CD4 Tregs is observed in PPs and the small intestine (Fig. 1D). Both Nrp-1− and Nrp-1+ CD4 Tregs comprised Ly-6C− and Ly-6C+ cells, suggesting that Ly-6C cannot be used to discriminate between iTregs and nTregs (Fig. 1E). Of note, among Nrp-1− CD4 Tregs, the proportion of Ly-6C− expressing cells gradually decreased from secondary lymphoid organs to the environmental surface represented by the small intestine.

Lack of Ly-6C expression in peripheral CD4 Tregs relies on TCR signaling events

We hypothesized that Ly-6C surface levels on peripheral CD4 Tregs might predict their propensity to interact with self-MHC class II molecules. Consistent with our hypothesis, we observed that Ly-6C− CD4 Tregs exhibited significantly higher densities of CD5 compared with their Ly-6C+ counterparts (Fig. 2A). In line with the greater capacity of Ly-6C− CD4 Tregs to interact with self, we found that the extent of CD3ζ phosphorylation was greater in Ly-6C+ CD4 Treg cells when compared to both Ly-6C− CD4 Tregs and naive CD4 T cells (pζ/ζ ratio, Fig. 2B).

TCR signals induce phosphorylation cascades, leading, in particular, to the relocalization of peculiar transcription factors. Although some of them move from the cytoplasm to the nucleus, others are excluded from the nucleus upon activation. Following stimulation, NFAT dephosphorylation leads to its translocation into the nucleus (21). In line with a role for TCR signaling in the modulation of Ly-6C expression in CD4 Tregs, we found that the proportion of nuclear NFAT2 was augmented in Ly-6C− CD4 Tregs compared with Ly-6C+ CD4 Tregs (Fig. 2C, 2D). Interestingly, differences in NFAT2 localization between Ly-6C+ and Ly-6C− CD4 Tregs were abolished when cells were rested in vitro for 1 h before staining or when access to MHC class II molecules was blocked in vivo 48 h before recovering secondary lymphoid organs (Fig. 2D).
Finally, in line with a role for TCR signaling in the modulation of Ly-6C expression in CD4 Tregs, we found that in vitro TCR-stimulated Ly-6C⁺ CD4 Tregs downregulated Ly-6C expression and that, by 3 d of culture, the extent of Ly-6C downregulation was proportional to stimulation strength (Fig. 2E). Altogether, our results suggest that Ly-6C⁺ CD4 Tregs interact more strongly than do Ly-6C⁻ CD4 Tregs with MHC class II-expressing cells. These interactions primarily involved self-peptides in the pLNs and spleen because in these organs, the proportion of Ly-6C⁻ expressing cells among Tregs was not affected in GF mice compared with conventional animals (Fig. 2F). More Ly-6C⁺ CD4 Tregs were recovered from the small intestine of GF mice, but a smaller proportion of these cells was found in the PPs and mLNs, suggesting that microbiota-derived Ags play a role in the induction/trafficking of Ly-6C⁺ CD4 Tregs at environmental surfaces.

Ly-6C⁻ CD4 Tregs are preferentially retained within LNs

A greater ability of Ly-6C⁻ CD4 Tregs to interact with self should have consequences on their behavior in secondary lymphoid organs. We recently showed that interactions with MHC class II molecules retained Ly-6C⁻ naive CD4 T cells in LNs (10). Using the same protocol (Fig. 3A), we analyzed the phenotype of CD4 T cells remaining within LNs after entry blockade. Most CD4 T cells have egressed from LNs 24 h after entry blockade, (Fig. 3B). Interestingly, Tregs were greatly enriched in the remaining CD4 T cells (Fig. 3C). Moreover, almost all LN Tregs were Ly-6C⁻ (Fig. 3D). At that time, few naive CD4 T cells and Ly-6C⁺ CD4 Tregs remained in cLNs and mLNs. Interestingly, Tregs were greatly enriched in the remaining CD4 T cells (Fig. 3C). Moreover, 24 h after initiating the treatment, almost all LN Tregs were Ly-6C⁻ (Fig. 3D). At that time, few naive CD4 T cells and Ly-6C⁺ CD4 Tregs remained in cLNs and mLNs. In contrast, a significant proportion of Ly-6C⁺ CD4 Tregs was still present in the same LNs (Fig. 3E). The differences that we observed in the percentage of recovery between mLNs and cLNs are

**p < 0.01, ***p < 0.001. ns, not significant.

FIGURE 2. Ly-6C expression in CD4 Tregs correlates inversely with their ability to interact with self. (A) CD5 fluorescence graphs of Ly-6C⁻ CD4 Tregs, Ly-6C⁺ CD4 Tregs, and naive CD4 T cells from LNs of a representative C57BL/6 Foxp3-GFP mouse. MFIs are shown as mean ± SEM for six mice from two independent experiments. (B) Immunoblot analysis of CD3ζ protein levels (ζ) and of the extent of CD3ζ-chain phosphorylation (pζ) (left panel). Quantification (compared with levels observed in naive CD4 T cells) is indicated under each blot. The scatter plot represents the ratio pζ/ζ for naive CD4 T cells (●), Ly-6C⁻ CD4 Tregs (○), and Ly-6C⁺ CD4 Tregs (gray circles). Each symbol represents an individual sample. (C) Four-color staining of F-actin (Phalloidin, blue), DNA (DAPI, white), Foxp3 (red), and NFAT2 (green) of freshly isolated Ly-6C⁻ and Ly-6C⁺ CD4 Tregs. Original magnification 3100. (D) LN cells were isolated from C57BL/6 mice, treated or not with an anti–MHC II–blocking Ab (Y3P), and fixed in 4% paraformaldehyde immediately or after 1 h of resting in culture. CD4 Tregs were sorted by flow cytometry and stained for Ly-6C, NFAT2, and DNA (DRAQ5). NFAT2 nuclear localization was calculated as similarity score between NFAT2 and DRAQ5 intensities. Data are representative of one of three independent experiments. (E) Flow cytometry–sorted LN Ly-6C⁻ or Ly-6C⁺ CD4 T cells from C57BL/6 Foxp3-GFP mice were cultured for 3 d in the presence or absence of graded doses of soluble anti-CD3 Ab, IL-2, and irradiated APCs. Representative Ly-6C fluorescence graphs for gated CD4 T cells are shown. (F) Ly-6C fluorescence graphs of CD4 Tregs from the indicated organs are shown for a representative mouse. Proportions of Ly-6C⁺ T cells among CD4 Tregs recovered from pLNs, mLNs, spleen, PPs, and small intestine (SI) of C57BL/6 GF mice or C57BL/6 SPF littermate mice. Data are mean ± SEM for six mice from two experiments.

**p < 0.01, ***p < 0.001. ns, not significant.
consistent with a recent report by Mandl et al. (22), showing that the transit of T cells through mLNs was faster than their transit through pLN.

Several lines of evidence strongly suggest that the expression of Ly-6C by peripheral CD4 Tregs correlates inversely with their ability to interact with self and that these interactions preferentially retain Ly-6C<sup>+</sup> CD4 Tregs within LNs, increasing their transit time.

**Activated/effector CD4 Tregs are almost all comprised within Ly-6C<sup>−</sup> CD4 Tregs**

We then compared the expression of activation markers by Ly-6C<sup>−</sup> and Ly-6C<sup>+</sup> Tregs. We found that Ly-6C<sup>−</sup> CD4 Tregs expressed more surface amounts of CD44 and less CD45RB and CD62L than did both Ly-6C<sup>+</sup> CD4 Tregs and naive CD4 T cells (Fig. 4A).

Moreover, nearly all CD69-expressing Tregs were comprised within Ly-6C<sup>−</sup> CD4 Tregs (Fig. 4B). Activated/effector CD4 Tregs are almost all comprised within Ly-6C<sup>−</sup> CD4 Tregs (Fig. 4C).

**Relative transcript levels of Tbx21, Rora, Bcl-6, Irf4, Rorc, and Prdm1 genes were analyzed by quantitative RT-PCR in the indicated CD4 T cell subsets. Data are mean ± SEM for two independent experiments. (E) CXCRI expression by LN Ly-6C<sup>−</sup> CD4 Tregs, Ly-6C<sup>+</sup> CD4 Tregs, and naive CD4 T cells (left panel). Proportions (mean ± SEM) of CXCRI<sup>−</sup> cells among LN Ly-6C<sup>−</sup> CD4 Tregs, Ly-6C<sup>+</sup> CD4 Tregs, and naive CD4 T cells for six C57BL/6 Foxp3-GFP mice from two independent experiments (right panel). *p < 0.05, **p < 0.01, ***p < 0.001.
within the Ly-6C- fraction. In line with their activated phenotype, an significant proportion of Ly-6C- CD4 Tregs expressed Ki-67, a marker of proliferating cells (Fig. 4B).

In the last decade, several markers, including CD103, ICOS, and TNFRII, have been used to subdivide the CD4 Treg compartment (23–26). Interestingly, most CD103-, ICOS-, and TNFRII-expressing Tregs were comprised within Ly-6C- CD4 Tregs (Fig. 4C). More recently, it was found that subsets of CD4 Tregs, named effector Tregs (eTregs), express transcription factors typically associated with the differentiation of Tconv (27, 28). More precisely, CD4 eTregs expressing a single transcription factor would be specialized in the suppression of the Th cells expressing the same transcription factor. Thus, T-bet–, IRF4–, RORα–, and Bcl6-expressing Tregs would be specialized in controlling Th1, Th2, Th17, and CD4 follicular helper effector T cells, respectively. All of these transcription factors were expressed at higher levels in Ly-6C- CD4 Tregs than in both Ly-6C+ CD4 Tregs and naive CD4 T cells (Fig. 4D). Blimp-1 (prdm1), a master regulator of terminal B cell differentiation, was described to be expressed in Tregs and naive CD4 T cells (Fig. 4D). CXCR3 expression, which characterized eTregs controlling Th1 cell responses, was restricted to Ly-6C- CD4 Tregs (Fig. 4E). Finally, only Ly-6C- CD4 Tregs expressed KLRG1, which was described as characterizing terminally differentiated eTregs (29) (Fig. 4C).

Ly-6C+ CD4 Tregs exhibit poor suppressive capacities in vitro and in vivo

We then compared the suppressive capacities of Ly-6C- and Ly-6C+ CD4 Tregs. Ly-6C+ CD4 Tregs were less efficient than their Ly-6C- cell counterparts in inhibiting the proliferative response of CD4 Tconv to anti-CD3 stimulation (Fig. 5A, 5B). Indeed, they exhibit suppressive capacities only at high Treg/Tconv ratios. We then tested whether Ly-6C+ CD4 Tregs also were lacking suppressive capacities in vivo. Naive CD4 T cells were injected alone or together with Ly-6C- or Ly-6C+ CD4 Tregs into CD3ε−/− mice (Fig. 5C). T cell–deficient recipient mice injected with naive CD4 T cells alone developed a severe wasting disease over time (Fig. 5D) (30). Although Ly-6C- CD4 Tregs efficiently inhibited development of the disease, coinjection of Ly-6C+ CD4 Tregs did not rescue recipient mice from colitis. Accordingly, histological colitis scores (determined 7 wk after transfer by examination of colon sections) were quite similar whether naive CD4 T cells were injected alone or together with Ly-6C+ CD4 Tregs (Fig. 5E). In secondary lymphoid organs, expansion of naive CD4 T cells was controlled less efficiently by Ly-6C+ CD4 Tregs than by Ly-6C- CD4 Tregs (Fig. 5F, left panels). These differences did not mirror a differential expansion of Ly-6C+ or Ly-6C- Tregs in response to lymphopenia, because the absolute numbers of Tregs recovered from the secondary lymphoid organs of recipient mice were similar, irrespective of their initial Ly-6C expression (Fig. 5F, right panels).

FIGURE 5. In vitro and in vivo suppressive capacities of Ly-6C- and Ly-6C+ CD4 Tregs. (A) LN CD4 Tconv (GFP+ CD4 T cells) of C57BL/6 Foxp3-GFP mice were sorted by flow cytometry, labeled with CTV, and cultured alone (no Treg) or together with the indicated Tregs at various Treg/Tconv ratios (2:1, 1:1, 1:2, 1:4) in the presence of soluble anti-CD3 Ab and APCs. CTV fluorescence graphs of Tconv (CTV*), 64 h after the beginning of culture. CTV fluorescence graph in the absence of anti-CD3 stimulation is shown as control (filled graph). (B) Inhibition percentages of the suppressive capacities of Ly-6C- and Ly-6C+ CD4 Tregs were calculated, as described in Materials and Methods, and plotted. Circles, squares, and triangles represent individual experiments. (C-F) A total of 5 × 10^5 flow cytometry–sorted naive CD4 T cells from C57BL/6 Foxp3-GFP mice were transferred into C57BL/6 CD3ε−/− recipient mice, alone or together with 5 × 10^6 flow cytometry–sorted Ly-6C- or Ly-6C+ CD4 Tregs from C57BL/6 Foxp3-GFP mice. Seven weeks after transfer, pLNs, mLNs, and spleen were recovered separately and analyzed. (C) Experimental model. (D) Body weight of recipient mice was monitored up to 7 wk posttransfer, and percentages of initial body weight were calculated and plotted. (E) Colitis scores of recipient mice were assessed 7 wk after transfer. (F) Absolute numbers of CD4 T cells deriving from initially injected naive (CD45.1+, left panels) or regulatory (CD45.2+, right panels) CD4 T cells were assessed 7 wk after transfer and plotted. Each symbol represents an individual recipient mouse. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.
We then compared the phenotype of Ly-6C− and Ly-6C+ CD4 Tregs with respect to molecules known to play a role in their suppressive capacities. We tested CD25 because it allows Tregs to deprive responder cells of their own IL-2; CD39 and CD73, two ectoenzymes that catalyze the generation of adenosine; CTLA-4, which allows the depletion of costimulatory molecules at the cell surface of APCs; and PD-L1, which decreases T cell activation through interaction with its receptor (31). All of these molecules were upregulated in Ly-6C− CD4 Tregs compared with their Ly-6C+ cell counterparts (Fig. 6A). Importantly, Foxp3 expression itself was similar in Ly-6C− and Ly-6C+ CD4 Tregs (Fig. 6A). Unlike Ly-6C− CD4 Tregs, Ly-6C+ CD4 Tregs did not transcribe several genes encoding for soluble factors (EBI3, IL-10, granzyme B, galectin-1, and TGF-β) involved in the suppression of immune responses more efficiently than naive CD4 T cells (Fig. 6B). At the protein level, we confirmed that Ly-6C− cells were the sole Tregs producing IL-10 upon activation (Fig. 6C). Of note, although they did not produce IL-10, Ly-6C+ CD4 Tregs were still unable to produce IL-2, IL-17, or IFN-γ (Fig. 6C).

Rapid decline in peripheral Ly-6C+ CD4 Treg numbers with age

We then decided to study the impact of ageing on the homeostasis of peripheral CD4 Tregs with respect to their expression of Ly-6C. The proportion of Ly-6C− CD4 Tregs was fixed rapidly after birth. However, most Ly-6C−-expressing CD4 Tregs from 1–2-wk-old mice exhibited lower surface levels of this marker compared with adult mice (Supplemental Fig. 2). This may reflect the acquisition of Ly-6C expression by recent thymic migrants, because a vast majority of Foxp3-expressing CD4 thymocytes do not express Ly-6C (Fig. 1A). Interestingly, the proportion of Tregs expressing Ly-6C decreased with age, and almost no Ly-6C+ CD4 Tregs were detected in the secondary lymphoid organs of 18-mo-old mice (Fig. 6A). More specifically, we found that absolute numbers of both naive CD4 T cells and Ly-6C+ CD4 Tregs decreased slowly in LNs and even increased with age in the spleen.

Ly-6C+ CD4 Treg disappearance with age could either reflect their death or Ly-6C downregulation. To address this issue, we first verified that Ly-6C expression on CD4 Tregs was almost stable (at least for 4 wk) after transfer into lymphoreplete recipient mice (Fig. 7C, 7D). Of note, under these conditions, almost all recovered Ly-6C− and Ly-6C+ cells were still expressing Foxp3. As we described (18, 32), injection of CD4 T cells into lymphopenic recipient mice leads to the activation and strong proliferation of part of the injected cells in response to self and environmental Ags, a process known as spontaneous proliferation. Under these conditions, when injected alone, some CD4 Tregs lose Foxp3 expression and strongly proliferate in response to lymphopenia (15, 33, 34). In this study, we found that both Ly-6C− and Ly-6C+ CD4 Tregs gave rise to Foxp3− CD4 T cells in response to lymphopenia and that nearly all recovered cells were Ly-6C− (Supplemental Fig. 3A, 3B). Interestingly, Ly-6C+ CD4 Tregs gave rise to higher proportions and absolute numbers of Foxp3− cells than did Ly-6C− CD4 Tregs (Supplemental Fig. 3C, 3D). This latter result may reflect a greater plasticity of Ly-6C+ CD4 Tregs. However, it also may derive from the greater ability of Ly-6C+ CD4 Treg–derived CD4 Tconvs to proliferate in response to lymphopenia.

In unmanipulated lymphoreplete mice, Ly-6C expression on CD4 Tregs may be lost with age as a result of changes in their environment. Thus, we purified Ly-6C− CD4 Tregs from 1- to 2-y-old mice and transferred them into 4-wk-old animals (Fig. 7E). Three weeks later, almost all transferred Tregs were still lacking Ly-6C expression (Fig. 7F). These results strongly suggest that changes in Ly-6C expression with age do not derive from differences between young versus aged environment, but rather reflect the death of Ly-6C− CD4 Tregs. In agreement with this hypothesis, Ly-6C− CD4 Tregs from 6–10-mo-old mice contained a higher proportion of apoptosis-prone cells than did their Ly-6C− cell counterparts, as revealed by the analysis of caspase-3 activity (Fig. 7G, 7H).

**FIGURE 6.** Ly-6C− CD4 Tregs express higher amounts of key immunosuppressive molecules. (A) Foxp3, CD25, CD39, CD73, CTLA-4, and PD-L1 fluorescence graphs of Ly-6C− CD4 Tregs, Ly-6C+ CD4 Tregs, and naive CD4 T cells from LNs of a representative C57BL/6 Foxp3-GFP mouse. (B) Transcript levels of Ebi3, Il10, Gzmb, Lgals1, and Tgfb1 genes were analyzed by quantitative RT-PCR for the indicated CD4 T cell subsets. Data are mean ± SEM for two independent experiments. (C) The proportions of IL-2−, IL-10−, IL-17−, and IFN-γ-producing cells among memory, naive, Ly-6C−, and Ly-6C+ CD4 Tregs recovered from LNs of C57BL/6 Foxp3-GFP mice were estimated after a pulse stimulation with PMA and ionomycin. Data are mean ± SEM for six mice from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
Finally, we purified Ly-6C\(^{-}\) and Ly-6C\(^{+}\) CD4 Tregs and transferred them into mice expressing or lacking MHC class II molecules (Fig. 7I). Four weeks later, after Ly-6C\(^{-}\) CD4 Treg injection, far more cells were recovered from MHC class II–competent recipient mice than from their MHC class II–deficient counterparts (Fig. 7I). In contrast, when Ly-6C\(^{+}\) CD4 Tregs were transferred, absolute numbers of recovered cells remained similarly low, regardless of whether recipient mice were expressing MHC class II molecules. Thus Ly-6C downregulation in CD4 Tregs depends on TCR signals, and survival of the resulting Ly-6C\(^{-}\) CD4 Tregs also relies on their ability to interact with MHC class II molecules.

Discussion

We recently showed that Ly-6C expression allows the division of the naive CD4 T cell compartment into two cell subsets with distinct self-reactivity (10). In this study, we show that peripheral CD4 Tregs are heterogeneous for Ly-6C expression, with ~30% of them expressing Ly-6C in secondary lymphoid organs. Ly-6C\(^{-}\) CD4 Tregs express more CD5 and exhibit a higher degree of CD3\(\zeta\) phosphorylation than do their Ly-6C\(^{+}\) counterparts. Moreover, Ly-6C\(^{-}\) CD4 Tregs are retained preferentially within LNs after entry blockade. Finally, the subcellular localization of the NFAT2 transcription factor differs between the two subsets, because Ly-6C\(^{-}\) cells exhibit an augmented proportion of nuclear NFAT2 that may reflect recent TCR signaling events. In agreement with this assumption, this difference is abolished when interactions with MHC class II molecules are blocked in vivo. Thus, altogether, our data strongly suggest that, among the peripheral CD4 Treg compartment, Ly-6C\(^{-}\) cells interact more strongly with MHC class II molecules than do Ly-6C\(^{+}\) cells.

In naive CD4 T cells, although impacting on their functionality, TCR signaling derived from self-recognition, known as tonic
signaling, falls below their activation threshold (35, 36). As shown previously by other investigators (37, 38), all CD4 Tregs show increased TCR triggering compared with naïve CD4 T cells, because both Ly-6C<sup>−</sup> and Ly-6C<sup>+</sup> CD4 Tregs exhibit stronger CD3ζ-chain phosphorylation (Fig. 2B) and express higher levels of surface CD5 (Fig. 2A) than do the bulk of naïve CD4 T cells. Furthermore, it is more likely that the TCR signals perceived by Ly-6C<sup>−</sup> CD4 Tregs are above their activation threshold, because these cells exhibit an activated phenotype, and a high proportion of them is cycling, as assessed by Ki-67 staining. These results are consistent with a report by Fisson et al. (39) showing that the Treg pool consists of activated proliferating cells in the steady-state. In LNs and spleen, the percentage of Ly-6C<sup>−</sup> cells does not vary whether mice are bred under GF conditions. Thus, although in tissues, particularly at environmental interfaces, a portion of Ly-6C<sup>−</sup> CD4 Tregs may be specific for commensal bacteria–derived peptides, self-peptides would be the main ligands recognized by these cells in LNs and spleen. Thus, two thirds of the CD4 Treg pool in secondary lymphoid organs corresponds to autoreactive cells perceiving signals from self-recognition above their activation threshold.

We showed recently that 5 d after transfer into recipient mice lacking the expression of MHC class II molecules, CD4 Tregs exhibit significantly diminished suppressive capacities (16). In this study, we extend these results by demonstrating that weakly self-reactive CD4 Tregs (Ly-6C<sup>−</sup>) can be characterized in the periphery of unmanipulated young mice and that such cells exhibit poor suppressive capacities in vitro and in vivo. Interestingly, Ly-6C<sup>−</sup> and Ly-6C<sup>+</sup> CD4 Tregs express similar amounts of Foxp3. Thus, although TCR signals are not required for maintaining Foxp3 expression in peripheral CD4 Tregs, they are mandatory for their suppressive abilities. Magg et al. (40) recently showed that human activated Tconv expressing low levels of Foxp3 do not display suppressive functions, because Foxp3 is primarily expressed in the cytoplasm and not in the nucleus. We did not observe such a differential subcellular localization of Foxp3 between Ly-6C<sup>−</sup> and Ly-6C<sup>+</sup> CD4 Tregs. Indeed, in both subsets, Foxp3 is found almost exclusively in the nucleus (Fig. 2C). Thus, Foxp3 nuclear expression, although necessary, is not sufficient to maintain the suppressive functions of peripheral Tregs. TCR signals in Ly-6C<sup>−</sup> CD4 Tregs lead to an import of Nfat2 into the nucleus. Differences in the subcellular localization of transcription factors between Ly-6C<sup>−</sup> and Ly-6C<sup>+</sup> CD4 Tregs may lead to a differential pattern of expression of various proteins, including key immunosuppressive molecules. For example, Nfat was shown to play an important role in CTLA-4 expression (41, 42). Thus, Nfat translocation into the nucleus in Ly-6C<sup>−</sup> CD4 Tregs may account for their enhanced expression of CTLA-4 compared with Ly-6C<sup>+</sup> CD4 Tregs. Thus, TCR signals may play a role in determining the suppressive capacities of Foxp3-expressing cells by modulating the activity of transcription factors other than Foxp3. However, we cannot rule out that the relationship between TCR signaling and CD4 Treg–suppressive capacities partially depends on Foxp3 transcriptional activity itself. Indeed, several studies showed that posttranslational modifications of Foxp3, such as acetylation or phosphorylation, can modulate its function without affecting its expression or localization (43, 44).

In contrast to the peripheral naïve CD4 T cell compartment that gradually diminishes in number with age, the absolute number of CD4 Tregs in secondary lymphoid organs remains generally constant throughout life. More specifically, Ly-6C<sup>−</sup> CD4 Tregs are cycling cells whose half-life depends on interactions with self, because 4 wk after their transfer, far more Ly-6C<sup>−</sup> CD4 Tregs were recovered from the periphery of MHC class II–expressing recipient mice compared with recipient mice lacking the expression of these molecules. In contrast, Ly-6C<sup>+</sup> CD4 Tregs are primarily resting cells, and their survival does not depend on TCR-signaling events, because similar numbers of Ly-6C<sup>+</sup> CD4 Tregs were recovered 4 wk after transfer into recipient mice lacking or expressing MHC class II molecules. TCR signals may act directly on CD4 Treg survival and/or indirectly by boosting IL-2 production by peripheral Tconv. Indeed, IL-2 is known to play a crucial role in CD4 Treg homeostasis and to induce their proliferation (45). However, the results of our adoptive-transfer experiments revealed that only Ly-6C<sup>−</sup> CD4 Tregs are sensitive to the prosurvival consequences of self-recognition. As a result, they maintain their numbers with age in the periphery, whereas Ly-6C<sup>+</sup> CD4 Tregs gradually disappear. Thus, although quantitatively stable, the CD4 Treg pool is qualitatively enriched with age in highly self-reactive and suppressive cells.

Our recent studies demonstrated that Ly-6C expression allows the discrimination of weakly and highly self-reactive naïve CD4 T cells and Tregs in the periphery [(10) and this study]. Using this new marker, we obtained data strongly suggesting that naïve CD4 T cells with the highest avidity for self are prone to differentiate into iTregs and that self-recognition events are required to set up and/or maintain the suppressive capacities of CD4 Tregs. Thus, autoreactivity increases the iTreg differentiation potential of naïve CD4 T cells and maintains the suppressive program of Tregs in the periphery. These unexpected consequences of self-recognition reveal virtuous feedback loops that allow autoreactivity to control its potential harmful consequences, such as the anti-self responses that would lead to autoimmunity.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Gating strategy used to discriminate peripheral CD4 T-cell subsets. Dot plots illustrating the gating strategy used to define peripheral naive CD4 T cells (CD4 T naive) as Foxp3^+ CD25^- CD44^{low} CD4^+ CD8α^- CD3^+ cells, regulatory CD4 T cells (CD4 Treg) as Foxp3^+ CD4^- CD8α^- CD3^+ cells and memory CD4 T cells (CD4 T mem) as Foxp3^-CD44^{hi} CD4^- CD8α^- CD3^+ cells.
Supplemental Figure 2. Ly-6C Expression in splenic CD4 Treg cells gradually increases with age after birth. (A) Ly-6C fluorescence histograms of CD4 Treg cells recovered from the spleen of representative 1-, 2-, 3- and 4-week-old C57BL/6 mice. (B) Relative numbers of Ly-6C− CD4 Treg cells, Ly-6C+ CD4 Treg cells and naive CD4 T cells from the spleen of C57BL/6 mice were calculated by dividing the absolute numbers at all studied ages by the mean absolute number at 1 month. Results are shown as means ± SEM for at least 4 mice per age group.
Supplemental Figure 3. Plasticity potential of Ly-6C⁻ and Ly-6C⁺ CD4 Treg cells in a lymphopenic environment. 5x10⁴ flow-cytometry sorted Ly-6C⁻ or Ly-6C⁺ CD4 Treg cells from LNs of C57BL/6 Foxp3-GFP mice were injected i.v. into C57BL/6 CD3ε⁻⁻ mice. (A) Diagram illustrating the experimental model. (B) Representative Ly-6C/Foxp3 dot-plots are shown for gated CD4 T cells recovered from the LNs of representative recipient mice. (C) Proportions of Foxp3⁻ cells among the progeny of injected Ly-6C⁻ CD4 Treg cells and Ly-6C⁺ CD4 Treg cells in the spleen and LNs are shown as means ± SEM. (D) Absolute numbers of CD4 T cells recovered from the periphery (LNs + spleen) of recipient mice 8 weeks after transfer. Results are shown as means ± SEM for 3 mice per group.
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