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CD2 Costimulation Reveals Defective Activity by Human CD4^{+}CD25^{hi} Regulatory Cells in Patients with Multiple Sclerosis

Clare M. Baecher-Allan[*,†], **Cristina M. Costantino**[*], **Gregory L. Cvetanovich**[*], **Charles W. Ashley**[*], **Gaëlle Beriou**[*], **Margarita Dominguez-Villar**[*,‡], and **David A. Hafler**[*,‡]

[*]Division of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

[†]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

[‡]Department of Neurology and Immunobiology, Yale School of Medicine, New Haven, CT 06520

Abstract

Studying the activity of homogeneous regulatory T cell (Treg) populations will advance our understanding of their mechanisms of action and their role in human disease. Although isolating human Tregs exhibiting low expression of CD127 markedly increases purity, the resulting Treg populations are still heterogeneous. To examine the complexity of the Tregs defined by the CD127 phenotype in comparison with the previously described CD4^{+}CD25^{hi} subpopulations, we subdivided the CD25^{hi} population of memory Tregs into subsets based on expression of CD127 and HLA-DR. These subsets exhibited differences in suppressive capacity, ability to secrete IL-10 and IL-17, *Foxp3* gene methylation, cellular senescence, and frequency in neonatal and adult blood. The mature, short telomere, effector CD127^{lo}HLA-DR^{+} cells most strongly suppressed effector T cells within 48 h, whereas the less mature CD127^{lo} HLA-DR^{-} cells required 96 h to reach full suppressive capacity. In contrast, whereas the CD127^{+}HLA-DR^{-} cells also suppressed proliferation of effector cells, they could alternate between suppression or secretion of IL-17 depending upon the stimulation signals. When isolated from patients with multiple sclerosis, both the nonmature and the effector subsets of memory CD127^{lo} Tregs exhibited kinetically distinct defects in suppression that were evident with CD2 costimulation. These data demonstrate that natural and not induced Tregs are less suppressive in patients with multiple sclerosis.

Regulatory T cells (Tregs) protect peripheral tissue from autoimmune inflammation by inhibiting the activation of self-reactive T cells that have escaped negative selection in the

Address correspondence and reprint requests to Dr. Clare M. Baecher-Allan, Harvard Medical School, 77 Avenue Louis Pasteur, HIM 650, Boston, MA 02115. callan@rics.bwh.harvard.edu.

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Disclosures

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thymus (1, 2). Shown to be essential for maintaining peripheral tolerance in mouse models (3), Tregs have been found to be dysfunctional in human autoimmune diseases such as multiple sclerosis (MS), type 1 diabetes, psoriasis, rheumatoid arthritis, and myasthenia gravis (4–8). The transcription factor Foxp3 is the most specific marker for mouse and human Tregs (9) and is expressed by ~5% of human peripheral blood $CD4^+CD45RO^+$ memory T cells that have been shown to exhibit regulatory function in vitro (10). Foxp3 is required for Treg function as mutations that reduce Foxp3 activity lead to autoimmunity in patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome and in scurfy mice (11, 12).

In humans, highly pure Treg populations have been difficult to isolate, as the majority of their surface Ag profile is shared with activated CD4 T cells. Thus, whereas human $CD4^+$ cells expressing high levels of CD25 are enriched for Treg activity, this $CD4^+$ $CD25^{hi}$ Treg population is both functionally and phenotypically heterogeneous. We previously demonstrated that human $CD4^+$ cells can be enriched for in vitro suppressor function on the basis of high expression of CD25 and that $CD25^{hi}$ Tregs from MS patients are less suppressive than those from healthy donors (6). Subsequent work has shown that the $CD25^{hi}$ Treg population is both functionally and phenotypically heterogeneous. For example, depending upon flow cytometric gating techniques for the $CD25^{hi}$ population, only ~85% of $CD25^{hi}$ Tregs express high levels of FOXP3 ex vivo (13). Yet the $CD25^{hi}$ cells that coexpress HLA-DR^+ are a homogenous Treg population that expresses high levels of FOXP3 but does not produce granzyme B or the suppressive cytokine IL-10 (14, 15). In contrast, whereas the HLA-DR^- Tregs express FOXP3 and exhibit in vitro suppressor function, they contain distinct populations of cells that can produce granzyme B and secrete IL-10 and IL-17 (1, 15).

Natural Tregs (nTregs) that are thymically derived and induced Tregs (iTregs) that arise from peripheral $CD4^+CD25^-$ cells may exhibit differences in function and stability, though both populations express FOXP3 and exhibit suppressive capacity. It is clear that low levels of CD127 (IL-7Rα–chain) expression identify $CD4^+CD25^+$ cells that express high levels of FOXP3 and exhibit in vitro suppressor activity (13, 16), and yet the data in these two seminal reports also demonstrated that a small population of $CD127^+$ cells express FOXP3. More recent studies examining the relationship between CD127 and FOXP3 expression have suggested that $CD127^{lo}$ does not define all FOXP3-expressing cells (17). Although iTregs and nTregs may not be discriminated by Foxp3 expression, they may differ in their propensity to transition from a suppressive cell to a proinflammatory IL-17–secreting cell (18). Furthermore, as Th17 cells are increased in patients with MS (19, 20), it is unexplored whether the population of Tregs that can convert to IL-17–secreting cells would be in a proinflammatory rather than a suppressive state when isolated from patients.

Treg activity can be altered by cytokines, differences in costimulatory signals, and modulating the strength of TCR signaling (21–23). In humans, both APCs and T cells express high levels of CD58 (LFA-3) that binds and signals through the CD2 receptor, whereas CD48 is the ligand for CD2 in the mouse (24). Although CD28 costimulation has been shown to be crucial for Treg development (23, 25), CD2 costimulatory signals appear to induce effector Tregs to exert an immediate suppressor activity (14). Allelic variants of

CD58 have been associated with increased risk of developing MS ($p < 10^{-11}$) and rheumatoid arthritis and with lower expression of CD58 (26, 27). CD2 signaling also favors the differentiation and maturation of IL-10–secreting T regulatory 1 Tregs and preferentially augments the expression of FOXP3 in human $CD25^{hi}CD127^{lo}$ Tregs as compared with CD28 costimulation (28). Finally, Marson et al. (29) demonstrated that Foxp3 actively binds to the CD2 locus and induces the transcription of CD2. Thus, whereas CD2 is not a commonly studied costimulatory molecule, there is growing evidence to suggest that it plays a significant role in human Treg activity. Thus, we examined the CD2 costimulatory pathway in the assays interrogating human Treg function.

In this study, we conducted a detailed analysis of the original $CD25^{hi}$ human Treg population by defining three subpopulations based on expression of CD127 and HLA-DR. We demonstrate that the $CD4^{+}CD25^{hi}$ population contains rapidly suppressive $FOXP3^{hi}$ $CD127^{lo}HLA\text{-}DR^{+}$ effector Tregs, and nonmature $FOXP3^{hi}$ $CD127^{lo}HLA\text{-}DR^{-}$ Tregs and a mixed population of $CD127^{+}HLA\text{-}DR^{-}$ cells that are $Foxp3^{int}$, but can produce IL-10 or IL-17, are not present in cord blood and are strongly suppressive when stimulated through CD2. Furthermore, although both populations of $CD127^{lo}$ Tregs exhibit complete *Foxp3* gene DNA hypomethylation, the $CD127^{+}CD25^{hi}$ cells reveal only partial *Foxp3* gene demethylation. In comparing the activity of these three $CD25^{hi}$ populations isolated from patients with MS and healthy donors, we found that both the $CD127^{lo}$ effector and nonmature Tregs but not the $CD127^{+}HLA\text{-}DR^{-}$ cells are defective in patients with MS when tested in in vitro assays given CD2 costimulation. These data show that the $CD4^{+}CD25^{hi}CD127^{lo}$ Tregs are defective in patients with MS, whereas the $CD127^{+}HLA\text{-}DR^{-}$ population that likely contains the subset of iTregs is functionally indistinguishable when isolated from healthy donors and patients with MS.

Materials and Methods

Subjects

Peripheral blood, drawn from healthy individuals and 20 subjects with relapsing–remitting MS, was obtained after informed consent with approval by the Institutional Review Board at the Brigham and Women's Hospital. Patients were between the ages of 20 and 60 y, had relapsing–remitting disease with Kurtzke Expanded Disability Status Scale scores between 0 and 2.5, and had not received any treatment for the past 3 mo. For the comparison study, samples were isolated from age- (within 5 y) and sex-matched healthy donors with no history of autoimmune diseases. Newborn cord blood was obtained from three healthy donors immediately after cesarean delivery. Births at which antibiotics were administered during labor/delivery and births to HIV-positive mothers were excluded.

Cell isolation

PBMCs were separated by Ficoll-Hypaque (GE Healthcare) gradient centrifugation. Total $CD4^{+}$ T cells were isolated via the $CD4^{+}$ T Cell Negative Isolation Kit II (Miltenyi Biotec), fluorochrome-labeled mAbs against CD62L (Dreg 56), CD25 (M-A251), CD45RO (UCHL1), HLA-DR (L243), CD73 (AD2) (all from BD Biosciences), and CD127 (R34.34) (Beckman Coulter), and sorted on a FACSAria (BD Biosciences) to typically >98% purity

in postsort analysis. T-depleted APC were isolated from PBMC by negative selection with anti-CD2 magnetic beads (Dynal) and irradiated with 3000 rad.

Cell culture reagents and Abs

Cells were cultured in 96-well U-bottom plates (Costar) in RPMI 1640 medium (BioWhittaker) supplemented as described previously (30) with 5% human AB serum (Cellgro Mediatech). To generate anti-CD3/CD2 beads, tosyl-activated beads (Dynal Biotech) were covalently bound with anti-CD3 (UCHT1) and anti-CD2 (BMA 0111; Dade Behring) mAbs at 1 μg/10^7 beads and used at 1 × 10^4 beads/well. Recombinant human IL-2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and human rIL-2 from Dr. Maurice Gately, Hoffmann-La Roche (31).

Flow cytometry analyses

Cells were fixed and made permeable using the eBioscience Foxp3 Staining Kit and stained for Foxp3 (206D from Biolegend or PCH101 from eBioscience) or isotype control. Samples were run on an FACSCalibur (BD Biosciences) using CellQuest software and analyzed using FlowJo software (Tree Star).

In vitro micrococulture

For ex vivo Treg assays, sorted cell populations were plated directly after ex vivo isolation: total autologous $CD4^+CD25^{med/lo}$ were used as responder T cells (Tresp) at 2.5 × 10^3/well and Tregs at 1.25 × 10^3/well, resulting in a 2:1 ratio. Ex vivo Treg populations and Treg clones were also stimulated at 1.25 × 10^3/well in the absence of Tresp to determine their ability to proliferate. The cultures were stimulated with anti-CD3/CD2 beads or plate-bound anti-CD3 (0.1 or 0.5 μg/ml) and irradiated T-depleted APCs. Proliferation was monitored on days 2 and 4 by replacing 100 μl media with media containing 1 μCi [^{3}H]thymidine (New England Nuclear) for 24 h. There was no proliferation when Treg populations were cultured alone, as they demonstrated levels of [^{3}H]thymidine incorporation that were always less than twice background, which was equivalent to <10% of the level of the Tresp early (day 2) proliferation values. Cytokine secretion in culture supernatants was analyzed by ELISA (IL-17A, DuoSet ELISA; R&D Systems) or by cytometric bead arrays (Human Th1/Th2 Cytokine kit II; BD Biosciences).

Generation of single-cell clones

Cells were sorted at one cell per well in X-Vivo 15 medium (BioWhittaker) containing 5% human serum and stimulated with soluble anti-CD3 (clone Hit3a; BD Biosciences) and anti-CD28 (both at 1 μg/ml), irradiated APC (1–5 × 10^4/well), and IL-2 (50 U/ml). After 30 d of expansion, each clone was tested for Foxp3 and HLA-DR expression, IL-17 and IL-10 production, and suppressive activity. To assess suppressive function, a portion of each clone was intensively washed to remove IL-2 and stimulated alone or in coculture as described above. Although $CD45RO^+$ Tregs can down-regulate Foxp3 expression after repeated stimulation (32), we found that clones stimulated in the presence of accessory cells retain ex vivo function and phenotype (1).

Gene methylation analysis

DNA methylation analysis was performed by bisulfite sequencing. Briefly, genomic DNA was isolated from T cell clones using the DNeasy blood and tissue kit (Qiagen). Sodium bisulfate treatment of purified genomic DNA was performed using the EpiTect bisulfite kit (Qiagen). PCR was performed as described previously (33). PCR products were purified using the QIA-quick PCR purification kit (Qiagen) and sequenced directly. Trace files were interpreted using 4Peaks (Mekentosj). Complete demethylation was indicated by >95% conversion of G to A in the sequenced product; sites of complete methylation were indicated when >95% of the sequence peak heights indicated G. All sequences that contained intermediate levels of both G and A were classified as partially methylated.

Telomere detection

Telomeres were detected by flow cytometry using fluorescence in situ hybridization (FISH) and a fluorescein-conjugated peptide nucleic acid probe (DakoCytomation). Relative telomere length was calculated as the ratio between the telomere signal of each sample and a Jurkat cell line as described (34).

Statistics

A standard two-tailed *t* test was used for statistical analysis; *p* values ≤0.05 were considered significant, except for the data generated from testing multiple conditions tested to examine the activity of Tregs isolated from healthy donors and patients with MS, for which significance was determined via one-way ANOVA analysis.

Results

CD127 and HLA-DR define three distinct populations of human CD25^{hi}Foxp3^{+} Tregs

Using flow cytometric analysis, CD4^{+} T cells were examined for the expression of CD127 and HLA-DR in relation to FOXP3, CD25, and CD45RO. The Tresp (CD4^{+}CD25$^{int/lo}$) population in each sample was used to discriminate CD127^{lo} from CD127^{+} expression levels. Of the fixed and nonviable FOXP3^{+} memory (CD45RO^{+}) Tregs, ~15% were CD127^{+}HLA-DR^{-}, ~15% were CD127^{lo}HLA-DR^{+}, and 70% were CD127^{lo}HLA-DR^{-} (Fig. 1*A*, 1*B*). Similar populations were identified when the viable CD25^{hi} memory Treg population was subgated via CD127 and HLA-DR expression (Fig. 1*C*, 1*D*). These data also demonstrate that isolating the viable CD25^{hi} population selectively isolates a portion of CD45RO^{+} memory Tregs.

To begin characterizing these three populations, the three subsets of CD25^{hi} Tregs that expressed different combinations of HLA-DR and CD127 were FACS sorted and analyzed for ex vivo Foxp3 expression. Although CD127^{lo}HLA-DR^{-} Tregs comprised the majority of Foxp3^{+} cells (Fig. 1*F*), the less abundant CD127^{lo}HLA-DR^{+} Tregs exhibited the strongest expression of FOXP3 (Fig. 1*E*, 1*G*). Furthermore, whereas only half of the CD127^{+}HLA-DR^{-} cells expressed FOXP3 and did so at lower levels than the other two populations (Fig. 1*E*, 1*G*), they expressed more FOXP3 than activated CD4^{+}CD25^{int} T cells (data not shown).

Human CD25^{hi} Treg subsets differ in suppressive ability

To examine whether these CD25^{hi} populations differed in suppressive ability, the different Treg subsets were FACS sorted and placed in coculture with CD4^{+}CD25^{-} Tresp under different stimulatory conditions. In the first assays, the cells were stimulated with anti-CD3/CD2 in the absence of APCs (Fig. 2*A*), a stimulus that was previously shown to promote rapid suppression by HLA-DR^{+} Tregs (14) and to initiate Tresp proliferation that can be detected as early as 48 h poststimulation (Supplemental Fig. 4). Under these conditions, all three ex vivo CD25^{hi} Treg subsets suppressed the proliferation of the cocultured Tresp cells, but with marked differences in the strength and kinetics of suppression (Fig. 2*A*). The CD127^{lo}HLA-DR^{+} Tregs exerted the strongest immediate suppressive activity, whereas the CD127^{lo}HLA-DR^{-} Tregs were less suppressive, and the CD127^{+}HLA-DR^{-} cells were not suppressive at the earliest stage of Tresp proliferation (day 2). However, when assayed later during maximal Tresp proliferation on day 4, both the CD127^{lo}HLA-DR^{-} and CD127^{+}HLA-DR^{-} subsets exhibited markedly increased suppressive function (for additional sample data, see Supplemental Fig. 4). These data indicated that both the CD127^{lo}HLA-DR^{-} and CD127^{+}HLA-DR^{-} subsets exhibited maturation of their suppressive effector function.

To examine whether T cell-depleted APCs affected suppressor function, the same CD25^{hi} subpopulations were placed in replicate cocultures with Tresp cells and stimulated with either anti-CD3/CD2 or varying concentrations of anti-CD3 with APCs. Consistent with the observation that strong TCR signals abrogate Treg suppression, all three CD25^{hi} subsets were unable to suppress Tresp activation in cocultures stimulated with high doses of anti-CD3, a condition known to promote IL-17 secretion (1 and data not shown). In the cocultures stimulated with lower dose anti-CD3, only the CD127^{lo}HLA-DR^{+} and CD127^{lo}HLA-DR^{-} cells significantly suppressed Tresp proliferation (Fig. 2*C*). In these cocultures, the inhibition of Tresp proliferation was also accompanied by the suppression of IL-10 secretion (Fig. 2*E*). Although the CD127^{lo}HLA-DR^{+} and CD127^{lo}HLA-DR^{-} cells exhibited similar ability to suppress proliferation and IL-10 production in response to stimulation with either anti-CD3/CD2 or anti-CD3/APC (Fig. 2*B*–*E*), the CD127^{+}HLA-DR^{-} cells only suppressed Tresp proliferation in response to anti-CD3/CD2 stimulation. All cocultures containing CD127^{+}HLA-DR^{-} cells exhibited marked increases in IL-10 secretion regardless of the effects on Tresp proliferation. In contrast, the cocultures established with the CD127^{+}HLA-DR^{-} cells and activated with anti-CD3 stimulation in the presence of APCs not only lacked suppression, but also exhibited marked increases in IL-17 secretion that were not seen with anti-CD3/CD2 stimulation (Fig. 2*F*, 2*G*). The distinct features of each clone are shown in Supplemental Fig. 1.

Foxp3-expressing CD127^{+}HLA-DR^{-} T cell clones are able to suppress and secrete IL-17

As CD127^{+}HLA-DR^{-} cells could either suppress or promote IL-17 secretion under different stimulation conditions and are isolated at low purity, as only ~50% of the ex vivo cells expressed Foxp3, we used single-cell cloning (14, 35) to investigate whether the population's divergent suppressive and IL-17 secretion activities could arise from an individual cell. As the Tregs that can express FOXP3 and IL-17 are known to reside in the CD25^{hi}HLA-DR^{-} population (1), we asked whether the Tregs that exhibited this functional

plasticity resided in the subpopulation of the Tregs that expressed CD127. To this end, we generated and analyzed 140 single-cell clones from the three $CD25^{hi}$ subpopulations.

After 30 d of expansion, the clones were examined and found to express FOXP3 at levels that correlated with the FOXP3 expression in their original ex vivo subset. As expected (14), the $CD127^{lo}HLA\text{-}DR^{+}$ subset exhibited extremely low cloning efficiency with an average efficiency of 3%, as the majority of experiments did not result in any $CD127^{lo}HLA\text{-}DR^{+}$ clones. Overall, the cloning efficiencies for the different $CD25^{hi}$ populations (Fig. 3*A*, *top panel*) inversely correlated with the intensity of FOXP3 expression. Furthermore, the relationship whereby the ex vivo $CD127^{+}HLA\text{-}DR^{-}$ cells expressed significantly higher levels of Foxp3 than ex vivo Tresp ($CD25^{lo}$) cells was still apparent in the resting clones (Fig. 3*A*, *bottom panel*), suggesting that the intermediate level of FOXP3 expressed by $CD127^{+}CD25^{hi}$ cells was maintained in the resting clones and was not merely a result of recent in vivo activation.

To examine whether the in vitro activities of the clones recapitulated the activities of the different ex vivo subpopulations, the clones were examined for their ability to secrete IL-10 or IL-17 when stimulated alone (Fig. 3*B*, 3*C*) and to regulate the proliferation and cytokine secretion of cocultured Tresp (Fig. 3*D*, 3*E*). Closely mirroring their corresponding ex vivo population, the clones derived from the $CD127^{+}$ or $CD127^{lo}$ subsets of $CD25^{hi}HLA\text{-}DR^{-}$ cells differed in their ability to suppress and induce IL-17 production when cocultured with Tresp. All of the $CD127^{lo}HLA\text{-}DR^{-}$-derived clones suppressed Tresp proliferation in cocultures stimulated with anti-CD3/CD2 (Fig. 3*D*). Furthermore, the majority of the $CD127^{lo}HLA\text{-}DR^{-}$-derived clones still exhibited some suppressive activity in the strong anti-CD3/APC-stimulated cocultures and did not result in the secretion of IL-17 (Fig. 3*E*). In contrast, whereas ~40% of the $CD127^{+}HLA\text{-}DR^{-}$-derived clones suppressed Tresp proliferation in response to anti-CD3/CD2 stimulation (7 out of 17 clones, Fig. 3*D*), three fourths of these clones secreted IL-17 when activated alone with strong anti-CD3/APC stimulation (Fig. 3*C*). As the $CD127^{+}HLA\text{-}DR^{-}$-derived clones secreted significant amounts of the inhibitory cytokine IL-10 in response to both stimuli (Fig. 3*B*, 3*C*), but only suppressed with anti-CD3/CD2 stimulation, and IL-10 neutralization had no effect on $CD127^{+}HLA\text{-}DR^{-}$ suppressive activity (data not shown), IL-10 does not appear to play a direct role in their suppressive mechanism. (The different functional parameters exhibited by each clone derived from the $CD127^{+}HLA\text{-}DR^{-}$ population are shown in Supplemental Fig. 1.) The capacity of the two ex vivo-sorted $CD25^{hi}HLA\text{-}DR^{-}$ populations ($CD127^{+}$ and $CD127^{lo}$) and respective clones to secrete IL-17 and IL-10 with or without FOXP3 expression in response to anti-CD3/CD2 or anti-CD3/CD28 stimulation was also examined by intracellular staining. The data shown in Fig. 4 indicate that although CD2 costimulation preferentially induces IL-10 secretion in both Treg populations, IL-17 secretion by the $CD127^{+}FOXP3^{+}$ cells was enhanced by CD28 costimulation. Furthermore, by focusing only on the $FOXP3^{+}$ cells, it is apparent that different cells are responsible for IL-10 and IL-17 secretion in both the ex vivo populations and the Treg clones.

Treg production of IL-17 is associated with CD73 expression and partial Foxp3 gene DNA methylation

Due to the heterogeneity of the $CD127^{+}HLA\text{-}DR^{-}$ population, a panel of Treg-surface molecules (36) was screened in an attempt to identify a marker that could distinguish cells able to both express FOXP3 and produce IL-17. An association between CD73 expression and IL-17 secretion was examined because the ex vivo $CD127^{+}HLA\text{-}DR^{-}$ population exhibited greater expression of CD73, an ecto-5′-nucleotidase active on mouse Tregs (37), than the IL-17 nonproducing $CD127^{lo}$ populations (Fig. 5*A*).

To examine whether CD73 expression was associated with IL-17 production by $CD127^{+}HLA\text{-}DR^{-}$ cells, we generated single-cell clones from $CD73^{+}$ or $CD73^{-}$ cells within the $CD127^{+}HLA\text{-}DR^{-}$ and $CD127^{lo}HLA\text{-}DR^{-}$ populations. The clones were activated with strong stimulation (anti-CD3/APC) to determine if they could produce IL-17 and express FOXP3 (1). As shown in Fig. 5*B–D*, although a number of clones derived from $CD127^{+}HLA\text{-}DR^{-}$ $CD73^{+}$ and $CD127^{+}HLA\text{-}DR^{-}CD73^{-}$ cells expressed FOXP3, only the clones derived from the $CD73^{+}$ cells secreted remarkably high levels of IL-17 (note that IL-17 is shown at nanograms per milliliter for these 11 out of 51 clones). The $CD127^{lo}HLA\text{-}DR^{-}CD73^{+}$ and $CD127^{lo}HLA\text{-}DR^{-}CD73^{-}$-derived clones expressed significantly higher levels of Foxp3 than the $CD127^{+}HLA\text{-}DR^{-}$-derived cells and did not secrete IL-17.

To determine if the functionally distinct clones exhibited unique Foxp3 gene methylation patterns, we examined the methylation state of the region in the first intron of *Foxp3*, referred to as the Treg-specific demethylated region (TSDR), in each clone. The level of Treg-specific demethylation has recently been shown to be a more specific and stable marker of human Tregs than Foxp3 expression (33, 38). The clones were functionally clustered by in vitro cytokine secretion, FOXP3 expression, and original expression of CD73. For comparison, the clones derived from Tresp population that could produce IL-2 with or without IL-17 exhibited full *Foxp3* methylation (Fig. 5*D*, representative two clones). The TSDR was completely demethylated in the Treg suppressive clones that were derived from the $CD127^{lo}HLA\text{-}DR^{-}$ population that were $FOXP3^{+}$ and unable to secrete either IL-17 or IL-2 (Fig. 5*D*, *top row*). In contrast, the TSDR was only partially demethylated in the suppressive clones that were derived from the $CD127^{+}HLA\text{-}DR^{-}CD73^{+}$ population that expressed FOXP3 and could secrete IL-17 but did not express IL-2 (Fig. 5*D*, *second row*). The TSDR methylation analysis of a larger panel of $CD127^{+}HLA\text{-}DR^{-}$ or $CD127^{lo}HLA\text{-}DR^{-}$-derived clones is shown in Supplemental Fig. 2 and indicates that high levels of FOXP3 can be expressed in $CD127^{+}HLA\text{-}DR^{-}$-derived clones that are heavily methylated in the *Foxp3* TSDR. The three ex vivo $CD25^{hi}$ populations exhibited similar TSDR methylation patterns (data not shown). The state of partial *Foxp3* demethylation in the $CD127^{+}HLA\text{-}DR^{-}$-derived clones that can suppress or secrete IL-17 might suggest that these cells are in an intermediate stage of unstable FOXP3 expression, possibly caught during their transition into a nonregulatory state.

Foxp3$^+$CD127^{lo}HLA-DR$^+$ and Foxp3$^+$CD127$^+$HLA-DR$^-$ cells are underrepresented in human neonatal blood

To determine whether the different CD25^{hi}FOXP3$^+$ populations may represent induced or nTreg populations, we investigated whether these Treg subsets existed in the neonatal circulation. As compared with their prevalence in adult peripheral blood, the different FOXP3-expressing HLA-DR and CD127 populations were examined in umbilical cord blood. In the neonatal circulation, the CD25$^+$ cells represented ~7% of the CD4$^+$ cells, and only 0.1% of them expressed HLA-DR (Fig. 6*C*). Thus, although the HLA-DR$^+$ Tregs were highly underrepresented in the neonate, they were FACS isolated and stained for FOXP3 and shown to again express the highest levels of FOXP3. In contrast, the CD127$^+$CD25$^+$ cells represented a much larger fraction of the neonatal CD4$^+$CD25$^+$ T cells, but these isolated cells completely lacked Foxp3 expression (Fig. 6*B–D*). Although all neonatal CD25$^+$ cells had been originally reported to express FOXP3 (39), our data agree with more recent reports demonstrating that a significant population of FOXP3$^-$CD25$^+$ cells reside in cord blood (40). The CD25$^+$CD127^{lo} population represented the largest fraction of FOXP3$^+$ cells in cord blood (Fig. 6*C*, 6*E*). It is important to note that as the majority of cord blood cells are naive and express CD45RA, the neonatal populations were isolated as CD25$^+$ rather than CD25^{hi}, and the majority of the isolated cells represent naive Tregs. Yet, the results indicate that, in the neonate, the FOXP3^{hi} CD25$^+$CD127^{lo}HLA-DR$^+$ effector population is rare but present, whereas the Foxp3$^+$ CD25$^+$CD127$^+$HLA-DR$^-$ cells are completely absent. In total, these data suggest that the CD127$^+$HLA-DR$^-$ Tregs are induced during immune system maturation.

Because HLA-DR$^+$ Tregs are rare in cord blood and do not undergo clonal expansion when isolated from adult peripheral blood, we examined whether the highly suppressive CD127^{lo}HLA-DR$^+$ Tregs are terminally differentiated. As short telomeres are a measure of cellular senescence (41, 42), flow-FISH methods of measuring telomere length were used to test this hypothesis. As shown in Fig. 6*F*, the telomeres of the CD127^{lo}HLA-DR$^+$ Tregs were on average 28% shorter than those of the CD127^{lo}HLA-DR$^-$ Tregs and 50% shorter than CD45RO$^+$ memory Tresp (Fig. 6*F*).

CD127^{lo}HLA-DR$^-$ and CD127^{lo}HLA-DR$^+$ Tregs exhibit distinct impairments of suppression in patients with MS

Finally, we examined the function of the three CD25^{hi} subpopulations isolated from patients with MS as compared with those isolated from healthy donors. Previous reports indicated that the CD25^{hi} Tregs from patients with relapsing–remitting MS exhibit impaired suppression (6, 43). However, more recent studies demonstrated that the Tregs isolated from patients with MS and healthy donors exhibit similar function when all memory CD127^{lo} Tregs are tested as a single population (44). Yet, as the HLA-DR$^+$ and HLA-DR$^-$ subsets both reside within the memory CD127^{lo} Treg population but exhibit distinct stimulation- and kinetic-dependent functions, we hypothesized that the distinct activities of each individual Treg subset may be obscured when assayed as a combined Treg population.

To compare the relative function of the different CD25^{hi} populations isolated from patients with MS and those from healthy donors, each CD25^{hi} population was isolated and

cocultured with Tresp cells in replicate assays that received different T cell activating stimuli. The three $CD25^{hi}$ populations isolated from patients with MS and healthy donors did not differ in frequency or intensity of FOXP3 expression, indicating similar Treg purity (Supplemental Fig. 3). The isolated cells were cultured alone or placed in cocultures with autologous Tresp cells and stimulated with either anti-CD3/CD2 or anti-CD3/APC (low and high doses, Fig. 7). The cocultures that received anti-CD3/CD2 stimulation were assayed for suppression of both immediate (day 2) and peak (day 4) proliferation, whereas suppression in the anti-CD3/APC-stimulated cocultures was measured at peak proliferation only (day 4). In the absence of Tresp cells, none of the $CD25^{hi}$ populations proliferated with stimulation (giving <200 cpm, data not shown). Importantly, this anti-CD3/APC stimulation was similar to the stimuli used in the recent reports demonstrating that patient-derived and healthy donor-derived Tregs exhibit identical suppressive function (44, 45).

As shown in Fig. 7, the patient- ($n = 20$) and control-derived ($n = 19$) $CD25^{hi}$ subpopulations exhibited identical suppressive capacity in cocultures given anti-CD3/APC stimulation but exhibited distinct kinetic differences in suppression in cocultures stimulated via CD2 (Fig. 7, Supplemental Fig. 4). Specifically, as compared with healthy donor cells, the patient-derived $CD127^{lo}HLA\text{-}DR^{-}$ cells were significantly less effective at inducing suppression on day 2 (Fig. 7*B*) in the cultures stimulated with anti-CD3/CD2. Yet, by the later time point, these patient-derived cells became as suppressive as those isolated from healthy donors. Conversely, although the $CD127^{lo}HLA\text{-}DR^{+}$ Tregs from both healthy controls and patients induced similar rapid ex vivo suppression on day 2, the patient-derived $CD127^{lo}HLA\text{-}DR^{+}$ Tregs were unable to maintain this level of suppression through day 4 (Fig. 7*A*). In contrast, the $CD127^{+}HLA\text{-}DR^{-}$ population from patients and healthy donors exhibited no differences in their ability to suppress at 4 d in response to CD2 costimulation (Fig. 7*C*).

Discussion

In this study, we identify three functionally distinct $CD25^{hi}$ populations that exhibit discrete in vitro suppressor function. Examination of Tregs isolated ex vivo and Treg clones demonstrates that these three populations exhibit differences in intensity of FOXP3 expression, ability to secrete IL-17, strength and kinetics of suppression, *Foxp3* gene methylation, CD73 expression, cellular senescence, and frequency in neonatal and adult blood. Specifically, coexpression of CD127 and CD73 appears to identify a $CD4^{+}CD25^{hi}$ subpopulation of iTregs capable of either in vitro suppressor function or proinflammatory IL-17 secretion depending upon the mode of costimulation, strength of TCR signal, and presence of Th17-inducing cytokines. Distinct from both $CD127^{lo}$ Tregs and effector CD4 cells, the $CD127^{+}CD25^{hi}$ cells are partially methylated in the first intron of *Foxp3*. Finally, we observed that the two $CD127^{lo}$ Treg populations were deficient in their ability to inhibit the proliferation of cocultured, autologous CD4 T cells when isolated from patients with MS and stimulated through CD2. These studies may provide insight into mechanisms that alter Treg function in human disease and may allow for the isolation of noninflammatory Treg populations for potential human clinical trials.

The similarities between the CD127^{lo}HLA-DR^{+} and CD127^{lo}HLA-DR^{-} Treg subsets suggest that these two populations share a common lineage and are distinct from the CD127^{+}HLA-DR^{-} population. Unlike the CD127^{+}HLA-DR^{-} cells, both CD127^{lo} subsets: 1) express high levels of FOXP3; 2) suppress in the presence of APCs; 3) are present in the neonate; 4) display a fully hypomethylated TSDR in the *Foxp3* gene; and 5) do not secrete IL-17. Due to these characteristics, the two CD127^{lo} Treg subsets likely represent thymically derived nTregs, whereas the CD127^{+}HLA-DR^{-} population, which is absent in the neonate and exhibits an increased capacity to coexpress FOXP3 and IL-17, likely contains induced, adaptive Tregs. The underrepresentation of CD127^{lo}HLA-DR^{+} Tregs in the neonatal compared with the adult circulation suggests that the presumed nTreg population gains HLA-DR expression upon differentiation. Indeed, naive CD4^{+}CD45RA^{+} Foxp3^{+} Tregs do not express HLA-DR. The persistent activation by self-Ag in the periphery could drive CD127^{lo} Tregs toward senescence and would account for both the reduced telomerase expression and increased cell turnover observed in CD4^{+}CD25^{hi} populations (46). Together with recent reports (15), these findings suggest that the CD127^{lo}HLA-DR^{+} Tregs are terminally differentiated, effector nTregs, as they do not proliferate, are highly sensitive to apoptosis (15), and are significantly more suppressive than the CD127^{lo}HLA-DR^{-} Tregs at the earliest possible, ex vivo time point (see day 2 assay of healthy donor-derived cells in Supplemental Fig. 4). In contrast, the CD127^{lo}HLA-DR^{-} Tregs are not terminally differentiated, as they can give rise to clones, and their suppressive capacity increases over time as indicated by comparing their in vitro suppressive capacity after 2 and 4 d in coculture (Supplemental Fig. 4).

It has recently been demonstrated that a subpopulation of both murine and human Tregs exhibits functional plasticity, as they can transition from suppressor to proinflammatory mediator associated with the secretion of IL-17 (1, 47). It has been proposed that this functional plasticity contributes to the increased immune activation in autoimmune diseases. These FOXP3^{+} cells that have the potential to secrete IL-17 also exhibit FOXP3 instability and can ultimately lose FOXP3 expression. However, it is clear that only a subpopulation of FOXP3^{+} Tregs exhibit this transcription factor instability with the potential for conversion to inflammatory function, leading to speculation that this functional plasticity may be more associated with induced FOXP3^{+} Tregs as compared with potentially more stable, thymically derived nTregs (48). Based on these functional properties and the inability to perform fate-mapping experiments in humans, we can only speculate that the FOXP3^{+} cells contained in the CD25^{hi}CD127^{+} population are adaptive Tregs. Thus, although it is critical to point out that the CD127^{+}HLA-DR^{-} subset is a mixed population, as only ~50% express FOXP3, this population is nevertheless able to suppress CD4 cells ex vivo. Furthermore, the study of single-cell clones derived from this population revealed that a number of the FOXP3^{+} cells in this population can either suppress in vitro or secrete IL-17, recapitulating the functional phenotype of the ex vivo CD127^{+}HLA-DR^{-} population (1). Within this population, the FOXP3^{+} IL-17–secreting T cells possessed incomplete methylation of the *Foxp3* TSDR, similar to murine TGF-β–treated iTregs (38). Although we were unable to find surface markers that would allow us to isolate a pure population of FOXP3^{+} and IL-17–inducible CD127^{+} cells, we were able to demonstrate that these cells were enriched in the population of cells that expressed CD73. CD73 is a cell-surface enzyme that converts

extracellular AMP into adenosine, which can signal through inhibitory receptors and attenuate lymphocyte activation and extravasation (49) and could be a mechanism by which these cells mediate inhibition.

In examining whether the Treg populations we identified had in vivo significance, we found that both of the natural $CD127^{lo}$ Treg subpopulations, but not the adaptive $CD127^{+}$ Treg population, exhibited defective suppressive activity in patients with MS. In response to CD2 costimulation, the patient-derived $CD127^{lo}$HLA-DR^{-} Tregs exhibited a significant reduction in their immediate suppressive activity on day 2, whereas the $CD127^{lo}$HLA-DR^{+} Tregs exhibited markedly less suppressor function on day 4. Interestingly, these same populations showed no deficit in suppression when stimulated with anti-CD3 and T cell-depleted APC. Thus, our findings are similar to the results reported by Michel et al. (44) and Venken et al. (45), who showed that the $CD4^{+}CD25^{hi}$ Tregs from patients with MS did not exhibit reduced suppressive activity when $CD127^{lo}$ Tregs were examined in coculture assays established with anti-CD3/APC stimulation (44, 45). However, as the patient-derived $CD127^{lo}$ Treg subsets exhibited defective suppression with CD2 costimulation, and CD58, which is the ligand for CD2, has been identified to be a risk factor for the development of both MS and rheumatoid arthritis (26, 27), it may be that the defect in Treg suppressive capacity involves the CD2 pathway of activation.

Our observation that patient-derived $CD127^{lo}$HLA-DR^{+} and $CD127^{lo}$HLA-DR^{-} Tregs have distinct defects in the kinetics of suppression suggests that there may be multiple underlying defective mechanisms and emphasizes the importance of separately studying these distinct Treg populations. Our data indicate that, as compared with cells from healthy donors, the patient-derived $CD127^{lo}$HLA-DR^{-} nonmature Tregs are less suppressive ex vivo, whereas the effector $CD127^{lo}$HLA-DR^{+} Tregs are less able to maintain suppression. To explain these observations, we propose that the $CD127^{lo}$HLA-DR^{-} Tregs exist in a less active state in the patient circulation, but can undergo normal effector maturation during the 4-d in vitro coculture. In contrast, although the effector $CD127^{lo}$HLA-DR^{+} cells isolated from patients with MS are highly suppressive upon isolation, they may exhibit a greater sensitivity to undergo apoptosis and thus an inability to sustain suppression (15) when isolated from patients. Therefore, the mechanisms underlying the defective suppression in patients with MS may be the result of two independent events: decreased in vivo functional maturation of $CD127^{lo}$HLA-DR^{-} and increased sensitivity to apoptosis by exhausted $CD127^{lo}$HLA-DR^{+} Tregs, perhaps mediated by granzyme B (15). This may provide a mechanism for the recently described decrease in suppressive activity of $CD39^{+}$ Tregs isolated from patients with MS (50), as all of the DR^{+} Tregs express CD39 (data not shown). Nevertheless, these data clearly indicate the need to examine functionally distinct populations of Tregs in human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

FISH	fluorescence in situ hybridization
iTreg	induced regulatory T cell
MS	multiple sclerosis
nTreg	natural regulatory T cell
Treg	regulatory T cell
Tresp	responder T cell
TSDR	regulatory T cell-specific demethylated region

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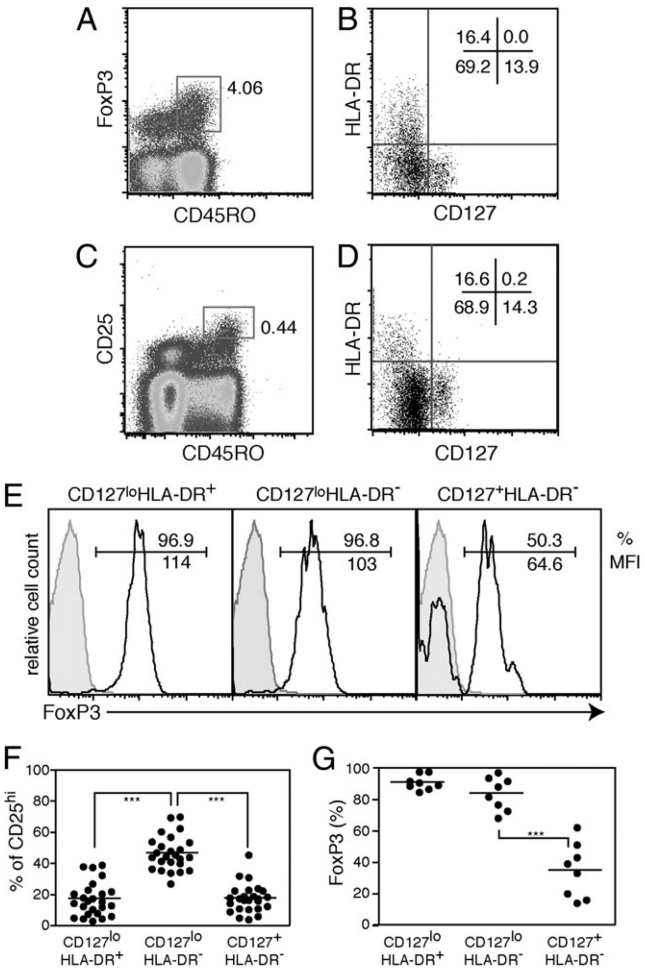

FIGURE 1.

Differential expression of CD127 and HLA-DR distinguishes three discrete populations of Foxp3$^+$ cells in the CD45RO$^+$ CD4$^+$ Treg compartment. Human peripheral blood CD4$^+$ T cells were stained for extracellular expression of CD45RO, HLA-DR, and CD127 and expression of CD25^{hi} or intracellular FOXP3. Tregs were identified by coexpression of Foxp3 and CD45RO (*A*) and analyzed for HLA-DR and CD127 expression (*B*). Tregs identified by bright CD25 expression (*C*) and analyzed for HLA-DR and CD127 expression (*D*). *E*, Intracellular FOXP3 staining of memory Treg populations sorted by coexpression of CD45RO and high levels of CD25 and HLA-DR or CD127, as indicated. *F*, Frequency of CD127^{lo} HLA-DR$^+$, CD127^{lo} HLA-DR$^-$, or CD127$^+$ HLA-DR$^-$ cells within the CD25^{hi} population. Each symbol represents an individual subject (n = 25 healthy donors, mean indicated; ***$p < 0.0001$). *G*, The frequency of FOXP3$^+$ cells within each CD25^{hi} Treg subpopulation (n = 8 healthy donors, mean indicated; ***$p = 0.0006$). MFI, mean fluorescence intensity.

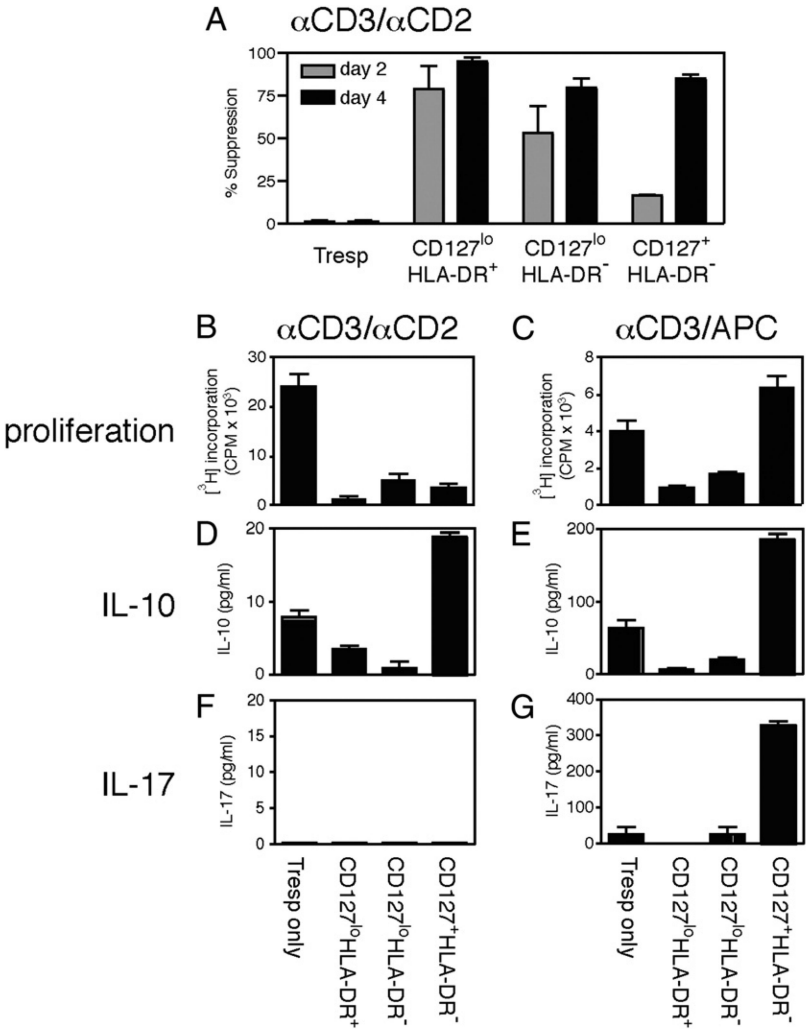

FIGURE 2.

CD127 and HLA-DR expression characterizes Treg subpopulations that exhibit stimulation-dependent and distinct functions. $CD4^+CD25^-$ $CD127^+$ Tresp were cultured alone or in a 2:1 ratio (Tresp/Treg) with FACS-purified $CD4^+CD25^{hi}$ $CD127^{lo}HLA\text{-}DR^+$, $CD127^{lo}HLA\text{-}DR^-$, and $CD127^+HLA\text{-}DR^-$ subpopulations. *A*, Percent suppression of proliferation in anti-CD3/CD2-stimulated cocultures at days 2 (gray) and 4 (black). No cytokine secretion was detected at the early time point. Cocultures stimulated using anti-CD3/CD2 or anti-CD3/APC were monitored for proliferation (*B*, *C*), and secretion of IL-10 (*D*, *E*), and IL-17 (*F*, *G*) on day 4.

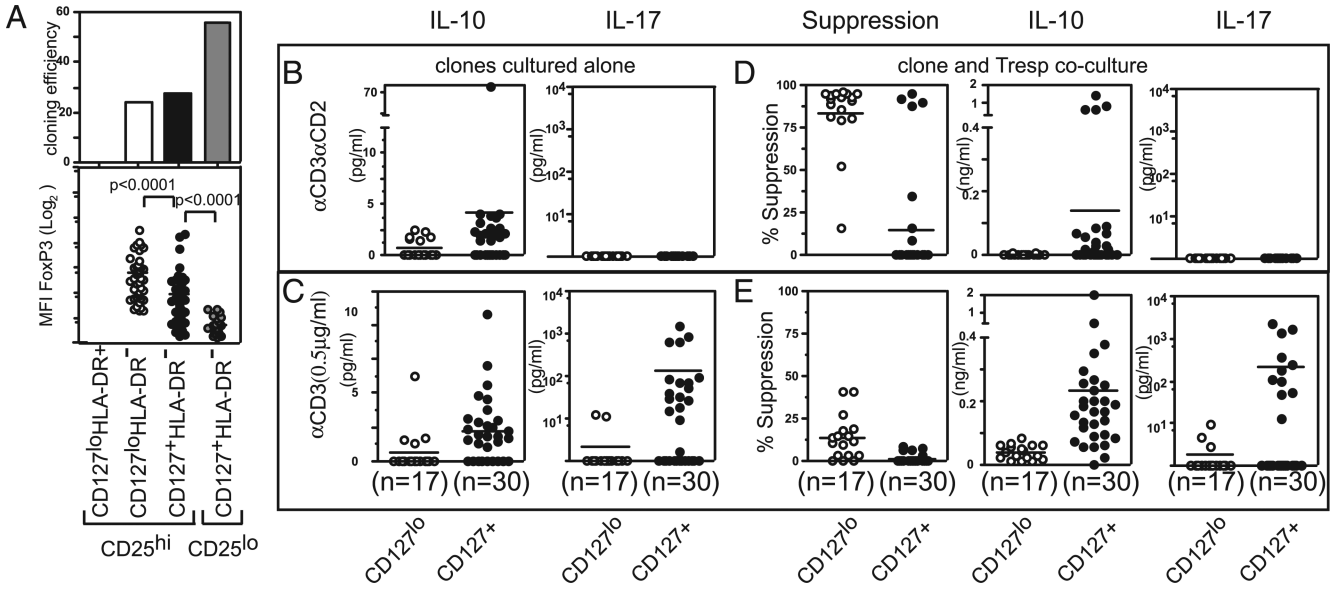

FIGURE 3.

Unlike CD127^{lo}-derived clones, the clones derived from the CD127^{+} subset can transition between IL-17 secretor and suppressor. Clones were generated via FACS sorting single cells from the three CD25^{hi} subpopulations. *A*, Clones were generated with the indicated cloning efficiency (*top panel*) and were stained for FOXP3 expression (*bottom panel*) when resting on day 30 poststimulation. The clones were stimulated either alone (*B*, *C*) or in coculture (*D*, *E*) with Tresp cells (2:1 Tresp/Treg) with anti-CD3/CD2 (*B*, *D*) or high-dose anti-CD3 (0.5 μg/ml) and APC (*C*, *E*). Suppression of proliferation was determined by [^{3}H]thymidine incorporation on day 4. IL-10 and IL-17 secretion was determined by cytokine bead array and ELISA. Each point represents a single clone; data representative of three independent cloning experiments.

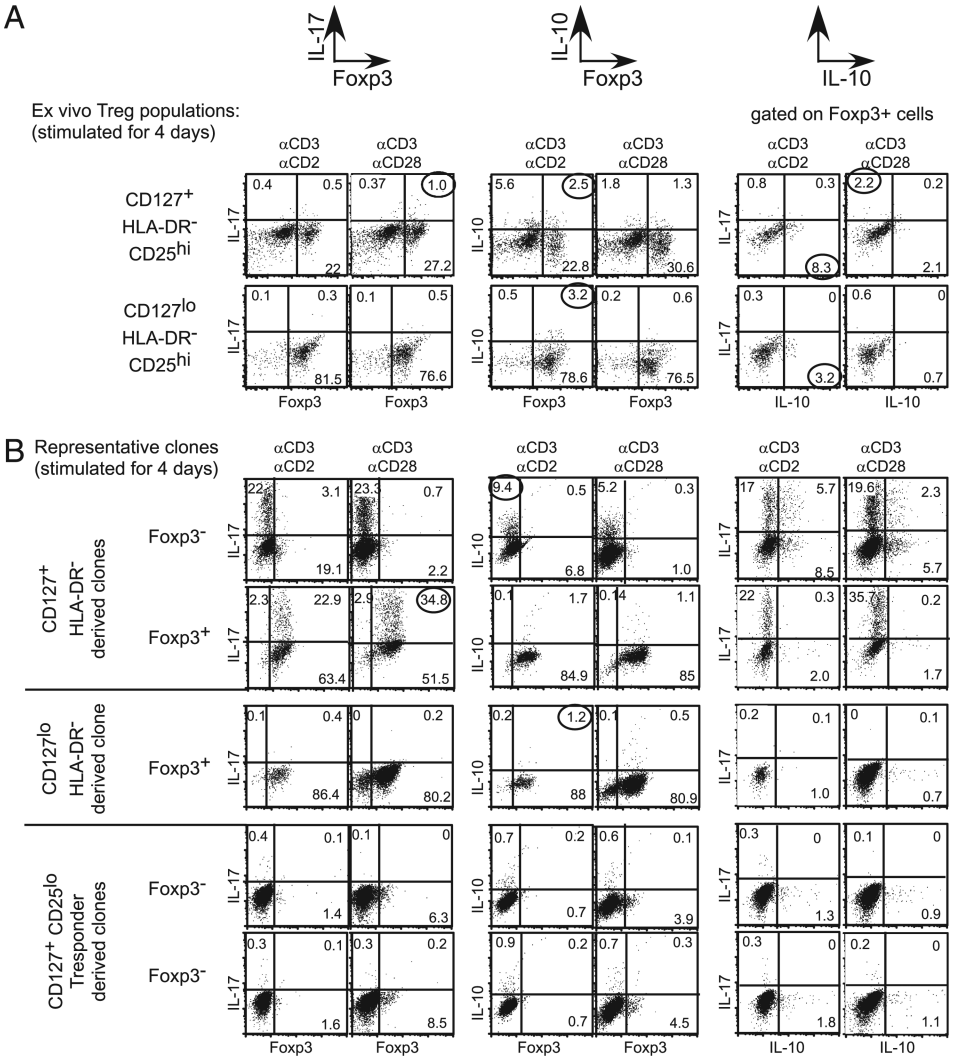

FIGURE 4.

CD2 and CD28 signaling differentially induces secretion of IL-17 or IL-10 by different FOXP3-expressing cells within the CD127^{+} population or CD127^{+}-derived clones. Sorted ex vivo CD127^{+}HLA-DR^{-} or CD127^{lo}HLA-DR^{-} populations of CD45RA^{-}CD25^{hi} cells (*A*) or clones derived from these populations (*B*) were stimulated with IL-2 (50 U/ml) and anti-CD3/CD2 or anti-CD3/CD28 for 4 d followed by a 4-h stimulation with PMA, ionomycin, and goligstop (BD Pharmingen) with subsequent intracellular staining for IL-17, IL-10, and FOXP3 (all mAbs obtained from eBioscience). The samples were run on an LSR II FACS analyzer (BD Pharmingen) and analyzed with FlowJo software (Tree Star). The major cytokine produced most prominently by the FOXP3^{+} cells in response to each pair of stimulations is circled.

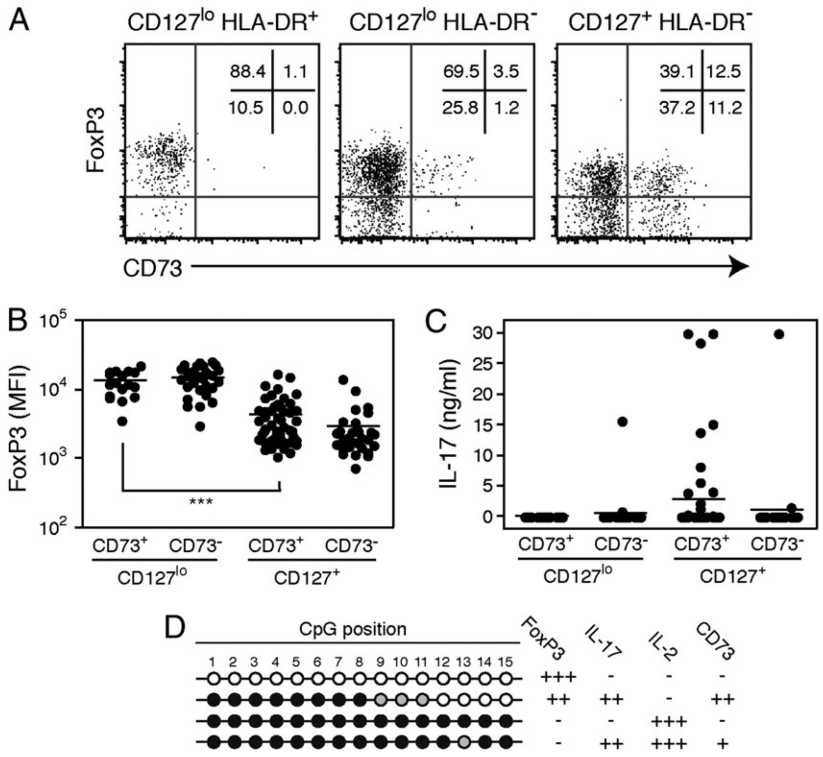

FIGURE 5.

FOXP3^{+} IL-17–producing CD25^{hi} CD127^{+} HLA-DR^{-} cells exhibit partial FoxP3 methylation and increased CD73 expression. *A*, Intracellular FOXP3 and extracellular CD73 expression on ex vivo CD25^{hi} Treg populations from a representative donor. *B* and *C*, Single-cell clones were generated from both CD73^{+} and CD73^{-} cells of the CD25^{hi} CD127^{lo} HLA-DR^{-} and CD25^{hi} CD127^{+} HLA-DR^{-} Treg subsets. After expansion, clones were stained for intracellular FOXP3 (***$p < 0.0001$) (*B*) and assayed for IL-17 production in response to proinflammatory stimulation (anti-CD3 at 0.5 μg/ml) by ELISA (*C*). Each point represents a single clone. *D*, CpG methylation of the Foxp3 TSDR and phenotype of four representative and functionally distinct clones. Each CpG position is represented as unmethylated (open), partially methylated (gray), or fully methylated (black).

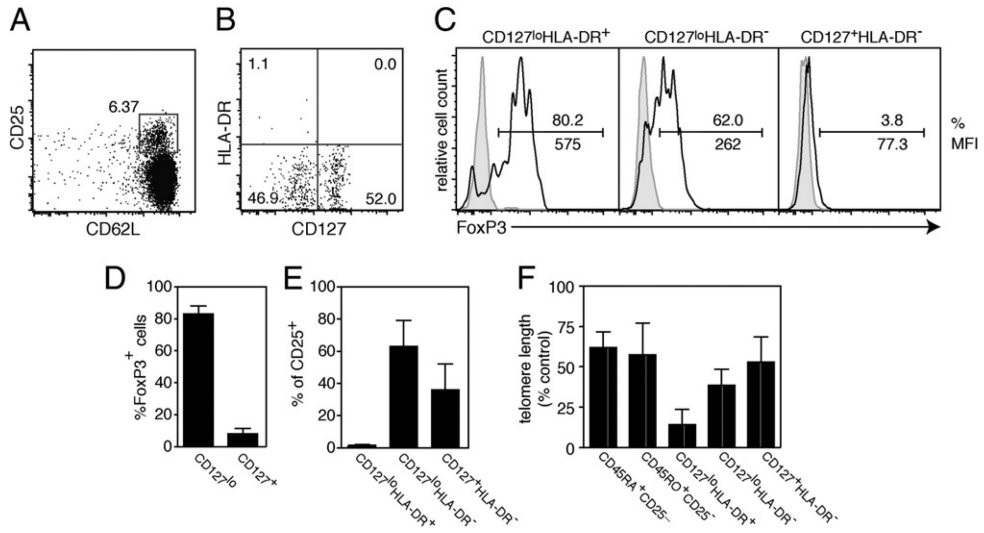

FIGURE 6.

The CD127^{+} HLA-DR^{-} and the CD127^{lo} HLA-DR^{+} FOXP3^{+} subpopulations are deficient in human cord blood. Human cord blood CD4^{+} T cells were stained for cell-surface CD25, CD62L, CD127, and HLA-DR. Cells coexpressing CD25 and CD62L (*A*) were analyzed for CD127 and HLA-DR expression (*B*). *C*, FOXP3 expression by CD127^{lo}HLA-DR^{+}, CD127^{lo} HLA-DR^{-}, and CD127^{+} T cells after FACS isolation from cord blood CD4^{+}CD25^{+}CD62L^{+} cells. *D*, Percentage of FOXP3^{+} cells in the CD127^{lo} and CD127^{+} subsets of CD4^{+}CD25^{+}CD62L^{+} from cord blood (n = 3; mean ± SD). *E*, Frequency of each cell subpopulation in the CD25^{+} compartment of cord blood (n = 3; mean ± SD). *F*, Telomere length was measured by flow-FISH in CD45RA^{+} and CD45RO^{+} CD25$^{-/lo}$ CD4^{+} T cells and in the three ex vivo CD4^{+}CD25^{hi} subpopulations after FACS isolation from adult PBMC. Data taken from seven healthy donors (mean ± SD) represented as the percentage of Jurkat telomeric length.

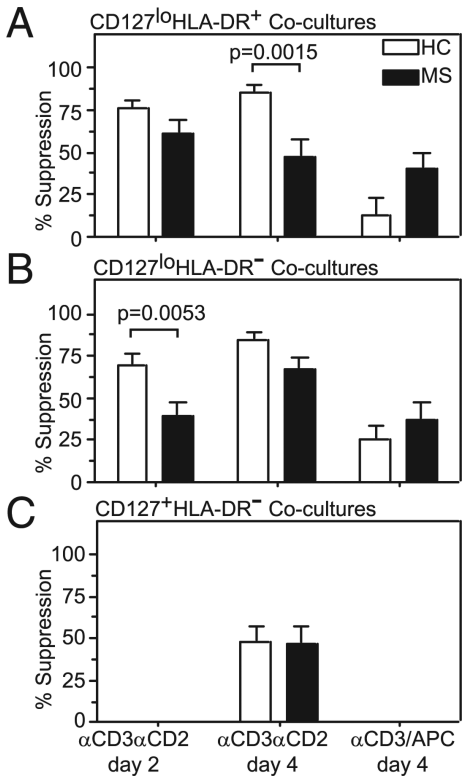

FIGURE 7.

Nonmature CD127^{lo}HLA-DR^{-} and effector CD127^{lo}HLA-DR^{+}CD25^{hi} Tregs exhibit distinct functional deficiencies in patients with MS. The three CD25^{hi} populations that are defined by expression of HLA-DR and CD127 were FACS sorted and placed in cocultures with autologous Tresp (2:1 Tresp/Treg). Cocultures were stimulated with anti-CD3/CD2 for 2 d (immediate) or 4 d, whereas those stimulated with anti-CD3 (0.5 μg/ml)/APC were assayed for proliferation on day 4. Capacity of the CD127^{lo}HLA-DR^{+} (**p = 0.0013) (*A*), CD127^{lo}HLA-DR^{-} (**p = 0.0061) (*B*), and CD127^{+}HLA-DR^{-} (*C*) cells to suppress the immediate or peak proliferation of the cocultured Tresp cells (CD4^{+}CD25$^{-/lo}$) is shown for cocultures of both healthy controls (open bars) and MS patients (filled bars). Each bar represents 15–20 individual (healthy donors or patients with MS) donors, SEM is indicated, and significance was determined by ANOVA statistical analysis.