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

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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\(^+\)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\(^{hi}\) 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.

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CD127lo cells express FOXP3. More recent studies examining the relationship between CD127 and FOXP3 expression have suggested that CD127lo 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 co-stimulatory 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 to 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 cells (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 CD25hiCD127lo human Treg population by defining three subpopulations based on expression of CD127 and HLA-DR. We demonstrate that the CD4+CD25hiCD127lo population contains rapidly suppressive FOXP3hiCD127lo HLA-DR− effector Tregs, and nonmature FOXP3hiCD127hiHLA-DR− Tregs and a mixed population of CD127hiHLA-DR− cells that are Foxp3hi, 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 CD127lo Tregs exhibit complete Foxp3 gene DNA hypomethylation, the CD127hiCD25lo cells reveal only partial Foxp3 gene demethylation. In comparing the activity of these three CD25hi populations isolated from patients with MS and healthy donors, we found that both the CD127lo effector and nonmature Tregs but not the CD127hiHLA-DR− cells are defective in patients with MS when tested in in vitro assays given CD2 costimulation. These data show that the CD4+CD25hiCD127lo Tregs are defective in patients with MS, whereas the CD127hiHLA-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 CD26L (Dreg 56), CD25 (M-2A51), CD45RO (UCHL1), HLA-DR (L243), CD73 (AD2) (all from BD Biosciences), and CD127 (R34.34) (Beckman Coulter), and sorted on a FACS(Aria) (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 (Cellogro Mediatech). To generate anti-CD3/CD2 beads, tosyl-activated beads (Dynal Biotech) were covalently bound with anti-CD3 (UCHT1) and anti-CD2 (BMA 0111; Dale Behring) mAbs at 1 μg/106 beads and used at 1 × 105 beads/well. Reconstituent 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 Biologic and PCH101 from eBio- science) 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+ CD25lo (1:1) APC were used as responder T cells (Tresp) at 2.5 × 105/well and Tregs at 1.25 × 105/well, resulting in a 2:1 ratio. Ex vivo Treg populations and Treg clones were also stimulated at 1.25 × 105/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 containing 1 μCi [3H]thymidine (New England Nuclear) for 24 h. There was no proliferation when Treg populations were cultured alone, as they demonstrated levels of [3H]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 UCHT1) and anti-CD2 (BMA 0111; Dale Behring) mAbs at 1 × 103 beads/well, resulting in 2.5 × 105/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 QIAquick 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
healthy donors and patients with MS, for which significance was deter-
multiple conditions tested to examine the activity of Tregs isolated from
were considered significant, except for the data generated from testing
CD127loHLA-DR+, and 70% were CD127loHLA-DR
CD127+HLA-DR
1
CD127+HLA-DR
CD25, and CD45RO. The Tresp (CD4+CD25int/lo) population in
each sample was used to discriminate CD127lo from CD127+
E
majority of Foxp3+ cells (Fig. 1
lating the viable CD25hi population selectively isolates a portion
of CD45RO+ memory Tregs.

To begin characterizing these three populations, the three subsets
of CD25hi Tregs that expressed different combinations of HLA-
and CD127 were FACS sorted and analyzed for ex vivo Foxp3 expression. Although CD127hiHLA-DR+ Tregs comprised the
majority of Foxp3+ cells (Fig. 1F), the less abundant CD127lo
Tregs exhibited the strongest expression of FOXP3 (Fig. 1E, 1G). Furthermore, whereas only half of the CD127hi
HLA-DR− cells expressed FOXP3 and did so at lower levels than
the other two populations (Fig. 1E, 1G), they expressed more
FOXP3 than activated CD4+CD25hi T cells (data not shown).

Human CD25hi Treg subsets differ in suppressive ability
To examine whether these CD25hi 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. 2A), 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 (Supple-
mental Fig. 4). Under these conditions, all three ex vivo CD25hi
Treg subsets suppressed the proliferation of the cocultured Tresp cells, but with marked differences in the strength and kinetics of suppression (Fig. 2A). The CD127hiHLA-DR+ Tregs exerted the strongest immediate suppressive activity, whereas the CD127lo
HLA-DR+ Tregs were less suppressive, and the CD127hiHLA-DR− Tregs were not suppressive at the earliest stage of Treg
proliferation (day 2). However, when assayed later during maximal Tresp proliferation on day 4, both the CD127hiHLA-DR− and
CD127hiHLA-DR− subsets exhibited markedly increased suppressive function (for additional sample data, see Supplemental
Fig. 4). These data indicated that both the CD127hiHLA-DR− and
CD127hiHLA-DR− subsets exhibited maturation of their suppressive effector function.

Results
CD127 and HLA-DR define three distinct populations of human CD25hiFoxp3+ 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+CD25hi) population in
each sample was used to discriminate CD127lo from CD127hi
expression levels. Of the fixed and nonviable FOXP3+ memory
(CD45RO+) Tregs, ~15% were CD127hiHLA-DR+, ~15% were
CD127loHLA-DR+, and 70% were CD127hiHLA-DR− (Fig. 1A,
1B). Similar populations were identified when the viable CD25hi
memory Treg population was subgated via CD127 and HLA-DR expression (Fig. 1C, 1D). These data also demonstrate that isolating the viable CD25hi population selectively isolates a portion
of CD45RO+ memory Tregs.

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 CD25hi 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 CD127hi HLA-DR−; CD127lo HLA-DR−, or CD127 hi
HLA-DR− cells within the CD25hi population. Each symbol represents an individual subject (n = 25 healthy donors, mean indicated; ***p < 0.0001). G, The frequency of FOXP3+ cells within each CD25hi Treg subgroup
(n = 8 healthy donors, mean indicated; ***p = 0.0006). MFI, mean fluorescence intensity.

To examine whether T cell-depleted APCs affected suppressor function, the same CD25hi 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 sup-
pression, all three CD25hi 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 CD127hiHLA-DR− and CD127loHLA-DR− cells significantly suppressed Tresp proliferation (Fig. 2C). In these cocul-
tures, the inhibition of Tresp proliferation was also accompanied by the suppression of IL-10 secretion (Fig. 2E). Although the
CD127hiHLA-DR− and CD127loHLA-DR− cells exhibited similar ability to suppress proliferation and IL-10 production in response

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 deter-
ined via one-way ANOVA analysis.

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).
to stimulation with either anti-CD3/CD2 or anti-CD3/APC (Fig. 2B–E), the CD127+HLA-DR− Tresp were cultured alone or in a 2:1 ratio (Tresp/Treg) with FACS-purified CD4+CD25hi CD127hiHLA-DR−, CD127loHLA-DR−, and CD127+HLA-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 2. CD127 and HLA-DR expression characterizes Treg subpopulations that exhibit stimulation-dependent and distinct functions. CD4+CD25hi CD127+ Tresp were cultured alone or in a 2:1 ratio (Tresp/Treg) with FACS-purified CD4+CD25hi CD127hiHLA-DR−, CD127loHLA-DR−, and CD127+HLA-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.

**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 CD25hiHLA-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 CD25hi 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 CD127hiHLA-DR− subset exhibited extremely low cloning efficiency with an average efficiency of 3%, as the majority of experiments did not result in any CD127hiHLA-DR− clones. Overall, the cloning efficiencies for the different CD25hi populations (Fig. 3A, top panel) inversely correlated with the intensity of FOXP3 expression. Furthermore, the relationship whereby the ex vivo CD127+HLA-DR+ cells expressed significantly higher levels of Foxp3 than ex vivo Tresp (CD25lo) cells was still apparent in the resting clones (Fig. 3A, bottom panel), suggesting that the intermediate level of FOXP3 expressed by CD127loHLA-DR− 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. 3B, 3C) and to regulate the proliferation and cytokine secretion of cocultured Tresp (Fig. 3D, 3E). Closely mirroring their corresponding ex vivo population, the clones derived from the CD127+ or CD127lo subsets of CD25hi HLA-DR− cells differed in their ability to suppress and induce IL-17 production when cocultured with Tresp. All of the CD127hi HLADR−-derived clones suppressed Tresp proliferation in their original ex vivo subset. As expected (14), the CD127hiHLA-DR− subset exhibited extremely low cloning efficiency with an average efficiency of 3%, as the majority of experiments did not result in any CD127hiHLA-DR− clones. Overall, the cloning efficiencies for the different CD25hi populations (Fig. 3A, top panel) inversely correlated with the intensity of FOXP3 expression. Furthermore, the relationship whereby the ex vivo CD127+HLA-DR+ cells expressed significantly higher levels of Foxp3 than ex vivo Tresp (CD25lo) cells was still apparent in the resting clones (Fig. 3A, bottom panel), suggesting that the intermediate level of FOXP3 expressed by CD127+HLA-DR− cells was maintained in the resting clones and was not merely a result of recent in vivo activation.

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derived clones suppressed Tresp proliferation in response to anti-CD3/CD2 stimulation (7 out of 17 clones, Fig. 3D), three fourths of these clones secreted IL-17 when activated alone with strong anti-CD3/APC stimulation (Fig. 3C). As the CD127\(^+\)HLA-DR\(^-\) derived clones secreted significant amounts of the inhibitory cytokine IL-10 in response to both stimuli (Fig. 3B, C), but only suppressed with anti-CD3/CD2 stimulation, and IL-10 neutralization had no effect on CD127\(^+\)HLA-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-DR\(^-\) population are shown in Supplemental Fig. 1.) The capacity of the two ex vivo-sorted CD25\(^+\)HLA-DR\(^+\) populations (CD127\(^+\) and CD127\(^-\)) 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 co-stimulation 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-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-DR\(^-\) population exhibited greater expression of CD73, an ecto-5'-nucleotidase active on mouse Tregs (37), than the IL-17 nonproducing CD127\(^+\) populations (Fig. 5A).

To examine whether CD73 expression was associated with IL-17 production by CD127\(^+\)HLA-DR\(^-\) cells, we generated single-cell clones from CD73\(^+\) or CD73\(^-\) cells within the CD127\(^+\)HLA-DR\(^-\) and CD127\(^+\)HLA-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. 5B–D, although a number of clones derived from CD127\(^+\)HLA-DR\(^-\)CD73\(^+\) and CD127\(^+\)HLA-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\(^+\)HLA-DR\(^-\)CD73\(^+\) and CD127\(^+\)HLA-DR\(^-\)CD73\(^-\) -derived clones expressed significantly higher levels of Foxp3 than the CD127\(^+\)HLA-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. 5D, representative two clones). The TSDR was completely demethylated in the Treg suppressive clones that were derived from the CD127\(^+\)HLA-DR\(^-\) population that were FOXP3\(^+\) and unable to secrete either IL-17 or IL-2 (Fig. 5D, top row). In contrast, the TSDR was only partially demethylated in the suppressive clones that were derived from the CD127\(^+\)HLA-DR\(^-\)CD73\(^+\) population that expressed FOXP3 and could secrete IL-17 but did not express IL-2 (Fig. 5D, second row). The TSDR methylation analysis of a larger panel of CD127\(^+\)HLA-DR\(^-\) or CD127\(^-\)HLA-DR\(^-\) -derived clones is shown in Supplemental Fig. 2 and indicates that high levels of FOXP3 can be expressed in CD127\(^+\)HLA-DR\(^-\) -derived clones that are heavily methylated in the Foxp3 TSDR. The three ex vivo CD25\(^+\) populations exhibited similar TSDR methylation patterns (data not shown). The state of partial Foxp3 demethylation in the CD127\(^+\)HLA-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**^\textsuperscript{hi} CD127^lo HLA-DR^+ and Foxp3^+ CD127^+ HLA-DR\textsuperscript{2} cells are underrepresented in human neonatal blood

To determine whether the different CD25^hi FOXP3\textsuperscript{+} 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. 6C). Thus, although the HLA-DR\textsuperscript{+} 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. 6B–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^hi population represented the largest fraction of FOXP3^+ cells in cord blood (Fig. 6C, 6E). 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^hi 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^hi HLA-DR\textsuperscript{+} effector population is rare but present, whereas the Foxp3^+ CD25^+ CD127^hi HLA-DR\textsuperscript{+} cells are completely absent. In total, these data suggest that the CD127^hi HLA-DR\textsuperscript{+} Tregs are induced during immune system maturation.

Because HLA-DR\textsuperscript{+} 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\textsuperscript{+} 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. 6F, the telomeres of the CD127^lo HLA-DR\textsuperscript{+} Tregs were on average 28% shorter than those of the CD127^lo HLA-DR\textsuperscript{+} Tregs and 50% shorter than CD45RO\textsuperscript{+} memory Tresp (Fig. 6F).

CD127^lo HLA-DR\textsuperscript{+} and CD127^lo HLA-DR\textsuperscript{+} 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^hi

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**FIGURE 4.** CD2 and CD28 signaling differentially induces secretion of IL-17 or IL-10 by different FOXP3-expressing cells within the CD127\textsuperscript{+} population or CD127\textsuperscript{hi} derived clones. Sorted ex vivo CD127\textsuperscript{+} HLA-DR\textsuperscript{−} or CD127\textsuperscript{hi} HLA-DR\textsuperscript{−} populations of CD45RA\textsuperscript{−} CD25\textsuperscript{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 goligostop (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\textsuperscript{+} cells in response to each pair of stimulations is circled.
Tregs are tested as a single population (44). Yet, as the HLA-DR+ and HLA-DR2 subsets both reside within the memory CD127lo 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 CD25hi populations isolated from patients with MS and those from healthy donors, each CD25hi population was isolated and cocultured with Tresp cells in replicate assays that received different T cell activating stimuli. The three CD25hi 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.
Conversely, although the CD127loHLA-DR+ Tregs from both patients and healthy donors ultimately became as suppressive as those isolated from healthy donors. CD3/CD2. Yet, by the later time point, these patient-derived cells exhibited distinct kinetic differences in suppression in 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.

As shown in Fig. 7, the patient- (n = 20) and control-derived (n = 19) CD25hi 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 CD127loHLA-DR− cells were significantly less effective at inducing suppression on day 2 (Fig. 7B) 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 CD127hiHLA-DR+ Tregs from both healthy controls and patients induced similar rapid ex vivo suppression on day 2, the patient-derived CD127loHLA-DR− Tregs were unable to maintain this level of suppression through day 4 (Fig. 7A). In contrast, the CD127hiHLA-DR+ population from patients and healthy donors exhibited no differences in their ability to suppress at 4 d in response to CD2 costimulation (Fig. 7C).

Discussion

In this study, we identify three functionally distinct CD25hi 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, CD3+ expression, cellular senescence, and frequency in neonatal and adult blood. Specifically, coexpression of CD127 and CD73 appears to identify a CD4+CD25hi 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 CD127hi Tregs and effector CD4 cells, the CD127hiCD25hi cells are partially methylated in the first intron of Foxp3. Finally, we observed that the two CD127lo 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 CD127hiHLA-DR− and CD127loHLA-DR− Treg subsets suggest that these two populations share a common lineage and are distinct from the CD127hiHLA-DR− population. Unlike the CD127hiHLA-DR− cells, both CD127lo subsets: 1) express high levels of FOXP3; 2) suppress in the presence of APCs; 3) are present in the neonate; and 4) display a fully hypomethylated TSDR in the Foxp3 gene; and 5) do not secrete IL-17. Due to these characteristics, the two CD127lo Treg subsets likely represent thymically derived nTregs, whereas the CD127hiHLA-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 CD127hiHLA-DR− Tregs in the neonatal compared with the adult circulation suggests that the presumed nTreg population gains HLA-DR expression upon differentiation. Indeed, naïve CD4+CD45RA+ Foxp3+ Tregs do not express HLA-DR. The persistent activation by self-Ag in the periphery could drive CD127lo Tregs toward senescence and would account for both the reduced telomerase expression and increased cell turnover observed in CD4+CD25hi populations (46). Together with recent reports (15), these findings suggest that the CD127hiHLA-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 CD127loHLA-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 CD127hiHLA-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 CD25hiCD127lo population are adaptive Tregs. Thus, although it is critical to point out that the
CD127loHLA-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 CD127loHLA-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 CD127lo 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 CD127loHLA-DR- Tregs exhibited a significant reduction in their immediate suppressive activity on day 2, whereas the CD127hiHLA-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 APCs. Thus, our findings are similar to the results reported by Michel et al. (44) and Venken et al. (45), who showed that the CD4+ CD25hi Tregs from patients with MS did not exhibit reduced suppressive activity when CD127lo Tregs were examined in coculture assays established with anti-CD3/APC stimulation (44, 45). However, as the patient-derived CD127lo 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 CD127loHLA-DR- and CD127hiHLA-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, compared with cells from healthy donors, the patient-derived CD127loHLA-DR- nonmature Tregs are less suppressive ex vivo, whereas the effector CD127hiHLA-DR+ Tregs are less able to maintain suppression. To explain these observations, we propose that the CD127hiHLA-DR- Tregs exist in a less active state in the patient circulation, but can undergo normal effector maturation during the 4-4d in vitro coculture. In contrast, although the effector CD127loHLA-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 CD127loHLA-DR- and increased sensitivity to apoptosis by exhausted CD127loHLA-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.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. The clones derived from the CD127+HLA-DR- population suppress with αCD3αCD2 stimulation but become IL-17 secreting, non-suppressors in response to strong TCR stimulation. The features associated with each clone given in Figure 3 are shown to demonstrate the capacity of clones to suppress under different stimulatory conditions, or transition into IL-17 secretors (in response to strong TCR stimulation with APCs). The Foxp3 expression, ability to secrete IL-17, and capacity to suppress is compared.
Supplemental Figure 2. By analyzing the methylation state of the Foxp3 intron (TSDR) in a larger panel of clones, it was found that CD127+ derived clones can express high levels of Foxp3 although the majority of their Foxp3 introns are heavily methylated. A larger panel of clones was examined for Foxp3 expression, capacity to produce IL-17 and IL-2, and methylation of the TSDR Foxp3 intron sequences using the bisulfite treatment/PCR sequencing method. Conversion of greater than 95% of the G residues to A residues was indicative of a non-methylated cytosine (open circle), while greater than 95% G residues indicated a highly methylated residue (filled circle), and gray circles indicate residues demonstrating intermediate levels of methylation.
Supplemental Figure 3. No phenotypic differences were detected in the CD25hi populations FACS sorted from healthy donors and patients with MS. CD4+ T cells were isolated from the peripheral blood of untreated patients with relapsing-remitting MS or healthy donors and stained for expression of cell surface CD25, CD127, and HLA-DR. (A) Frequency of total CD25hi cells and (B) each CD25hi subpopulation did not differ between healthy controls (HC, open rectangles) and MS patients (MS, filled rectangles). There was also no difference between the cells isolated from patients with MS and those from healthy donors with regards to (C) frequency of Foxp3 expression and (D) intensity of Foxp3 expression in the FACS sorted populations that were used the co-culture assays shown in Figure 6 (representing 15-20 patients with MS, and 15-20 healthy donors). Note: depending upon cell yield, all assays were not established with all CD25hi subsets (please see the legend to Supplemental figure 3 for the number of donor and patient cells assayed under each condition).
Supplemental Figure 4. The levels of $^3$H Thymidine incorporation indicate that patient and healthy donor Treg cells proliferated equally to the $\alpha$CD3xCD2 stimulation, and exhibited various levels of suppression when co-cultured with each of the three autologous CD4+CD25^{hi} Treg populations. $^3$H Thymidine incorporation in cultures of Treg cells established with and without the different CD25^{hi} populations is shown. The proliferation of cells isolated from healthy donors (n=20) and patients (n=20) with MS are shown as white and black filled circles, respectively. Not all assays and time-points could be analyzed for all cell types due to low cell yield. Thus the number of samples of control and patient cells (control/patient) that were analyzed for each CD25^{hi} subset was: 15/15 (day 2) and 18/16 (day 4) for CD127^{lo}HLA-DR^{+}, 19/19 (day 2) and 20/20 (day 4) for CD127^{lo}HLA-DR^{--}, and 19/20 (day 2) and 20/20 (day 4) for CD127^{hi}HLA-DR^{--}. The % suppression data shown in Figure 6 was derived from these data. $^3$H thymidine incorporation is shown for cultures stimulated with $\alpha$CD3xCD2 and pulsed on day 2 (A) or day 4 (B), or stimulated with $\alpha$CD3/APC and pulsed on day 4 (C). The level of early suppression induced by healthy donor CD127{lo}HLA-DR{+} and CD127^{lo}HLA-DR{--} populations was found to be significantly different by t-test analysis.