Human CD39$^+$ T$_{reg}$ Cells Express Th17-Associated Surface Markers and Suppress IL-17 via a Stat3-Dependent Mechanism

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Human CD4$^+$ T regulatory cells (T$_{regs}$) are a population of phenotypically and functionally diverse cells that downregulate inflammatory and autoimmune responses. As Th17 cells play an important role in the pathogenesis of autoimmune diseases, it is critical to elucidate the mechanisms regulating these cells. In this study, we examined the molecular basis underlying the phenotypic and functional diversity of human T$_{regs}$ expressing the ectonucleotidase CD39. CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ inhibit the proliferative response and the secretion of IL-17 and IFN-γ of autologous CD4$^+$ T effector cells, while CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ only suppress IFN-γ production. We demonstrate that activated human CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ express the Th17-associated surface markers CCR6 and IL-23R, and phosphorylate the transcription factor Stat3. Moreover, suppression of IL-17 by CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ occurs via a Stat3-dependent mechanism as inhibition of Stat3 activation in the CD39$^+$ T$_{regs}$ reverses their ability to suppress IL-17. CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ are not endowed with the ability to inhibit IL-17 as they do not upregulate CCR6 or the IL-23R, and furthermore, they secrete IL-17. Our findings provide the first evidence that human T$_{reg}$ functional diversity is matched to the type of immune response being regulated and reveal a new role for Stat3 in controlling T$_{reg}$ function.

Keywords: CD39$^+$ T$_{reg}$ cells, Stat3, IL-17

Introduction

HUMAN THYMUS-DERIVED naturally occurring T regulatory cells (T$_{regs}$) are an essential subset of CD4$^+$ T cells that maintain immune homeostasis, control immune responses to different pathogens, and prevent autoimmunity. T$_{regs}$ were initially described as a population of CD4$^+$ T cells expressing high levels of CD25 (CD25^{hi}) that suppressed the proliferation of CD4$^+$CD25^{neg} T cells *in vitro* (Sakaguchi and others 1995; Baecher-Allan and others 2001). The transcription factor Foxp3 was shown to be expressed predominantly by CD4$^+$CD25^{hi} T$_{regs}$ (Bennett and others 2001; Brunkow and others 2001; Wildin and others 2001; Fontenot and others 2003; Hori and others 2003; Bacchetta and others 2006). Identifying and isolating human T$_{regs}$ is challenging as expression of CD25 and Foxp3 increases transiently upon activation on CD4$^+$ T effector cells (T$_{effs}$) (Lipkowitz and others 1984; Morgan and others 2005). Foxp3 expression is intracytoplasmic and cannot be used for isolating T$_{regs}$. Other potential ''signature'' markers for human T$_{regs}$ include CTLA-4 (CD152) and CD27 (Dieckmann and others 2001; Jonuleit and others 2001; Koenen and others 2005; Ruprecht and others 2005) and lack of expression of CD127, the IL-7Rα (Liu and others 2006; Seddiki and others 2006; Hartigan-O'Connor and others 2007).

CD39 and CD73, ectonucleotidases responsible for hydrolysis of ATP to adenosine, are coexpressed on nearly all murine T$_{regs}$ (Borsellino and others 2007; Deaglio and others 2007). In humans, by contrast, CD39 expression is highly variable and restricted to a subset of CD4$^+$CD25^{hi} Foxp3$^+$ T$_{regs}$ (Borsellino and others 2007; Fletcher and others 2009; Mandapathil and others 2009). CD39 and CD73 coexpression is observed in a low percentage of human T$_{regs}$ (Dwyer and others 2010; Mandapathil and others 2010). The difference in expression of these ectonucleotidases between murine and human T$_{reg}$ cells underscores that extrapolation from murine to human T$_{reg}$ cells may not always be applicable. Furthermore, human, but not murine, Th17 cells were described as being resistant to T$_{reg}$ suppression (Annunziato and others 2002; Evans and others 2007). Interestingly, CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ are endowed with the ability to suppress IL-17 production by CD4$^+$ T$_{effs}$ (Fletcher and others 2009; Ye and others 2011).

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The Th17-associated chemokine receptor, CCR6, is expressed on human and mouse T_{regs} (Kleinewietfeld and others 2005; Acosta-Rodriguez and others 2007; Kluger and others 2014). $CCR6^+$ T_{regs} express Foxp3, exhibit T_{reg} functional properties, migrate in response to the CCR6 ligand CCL20, express memory cell markers, and produce IL-10. CCR6 was expressed on human T_{regs} that secrete IL-17 directly *ex vivo* (Ayyoub and others 2009) or on activation (Voo and others 2009).

The suppressive mechanisms used by T_{regs} to inhibit immune responses are complex and not completely understood. The suppressive mechanisms are cell–cell contact dependent and include the release of inhibitory cytokines (IL-10, TGF-β, or IL-35) (Nakamura and others 2001; Levings and others 2002; Li and others 2006, 2007; Collison and others 2007, 2009; Niedbala and others 2007; Chaturvedi and others 2011; Chaudhry and others 2011; Huber and others 2011), modulation of antigen-presenting cell function (CTLA-4) (Read and others 2000; Takahashi and others 2000; Oderup and others 2006; Onish and others 2008), cytolysis (granzymes and perforin) (Grossman and others 2004), generation of suppressive metabolites (adenosine) (Huang and others 1997; Fredholm and others 2003; Naganuma and others 2006), and metabolic disruption (consumption of IL-2) (de la Rosa and others 2004; Busse and others 2010). T_{regs} were also reported to inhibit Th17 cells via a latency-associated peptide (LAP)-dependent mechanism (Ye and others 2011). It is possible that T_{regs} use other mechanisms to inhibit specific T effector cell responses.

Several groups demonstrated that murine T_{regs} expressed canonical $CD4^+$ T effector cell-associated transcription factors to control polarized Th1-, Th2-, and Th17-driven immune responses (Chaudhry and others 2009; Koch and others 2009; Zheng and others 2009). Murine T_{regs} upregulate the expression of the transcription factors T-bet, IRF4, and GATA-3, and Stat3 to efficiently suppress Th1 (Koch and others 2009), Th2 (Zheng and others 2009), and Th17 responses (Chaudhry and others 2009), respectively. Similar lineage-specific T_{regs} are yet to be identified in humans.

In the present study, we describe a subset of human CD39-expressing $CD4^+CD25^{hi}Foxp3^+$ T_{regs} that upregulate expression of the Th17-associated chemokine receptor CCR6, upregulate the IL-23 cytokine receptor, and phosphorylate Stat3 on activation. These T_{regs} inhibit the proliferation, as well as IFN-γ and IL-17 production, of autologous $CD4^+$ T_{effs}. We demonstrate that suppression of IL-17 production by these cells occurs via a Stat3-dependent mechanism. This study suggests that $CD4^+CD25^{hi}CD39^+CCR6^+IL\text{-}23R^+Foxp3^+$ T_{regs} represent human Th17-specific regulatory T cells ($T_{reg}17$).

Materials and Methods

Isolation of peripheral blood mononuclear cells

Human subjects age 23–55 years old with no known autoimmune conditions were recruited from the campus of the University of Medicine and Dentistry of New Jersey-New Jersey Medical School (now Rutgers-NJMS) in Newark, NJ. Research protocols were approved by the Institutional Review Board of NJMS and performed in accordance with the regulatory guidelines. Written informed consent was obtained from each subject. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Histopaque gradient centrifugation of heparinized venous blood as described in Liu and Rohowsky-Kochan (2008).

Antibodies

The following fluorochrome-conjugated mouse monoclonal antibodies specific for human markers were used in this study. APC-Cy7-anti-CD4 (clone RPA-T4), Pacific Blue-anti-Foxp3 (clone 259D), APC-anti-CD39 (clone TU66), and Brilliant Violet 421 anti-CD73 (clone AD2) were obtained from BioLegend (San Diego, CA). PE-anti-CD25 (clone M-A251), Alexa Fluor700-anti-CD25 (clone M-A251), and PE-Cy5-anti-CD152 (CTLA-4, clone BNI3) were obtained from BD Pharmingen (San Jose, CA). APC-anti-CCR6 (clone #53103) and FITC-anti-IL-23R (clone #218213) were obtained from R&D Systems (Minneapolis, MN). LEAF™ purified anti-human CD3 (clone HIT3a) Abs from BioLegend were used for activation.

Surface and intracellular phenotyping of T_{regs}

Resting, nonactivated, or ''ex vivo isolated'' PBMC were examined for expression of surface CD4, CD25, CD39, CD73, CCR6, IL-23R, and CTLA-4 using fluorochrome-conjugated Abs. For intracellular staining, cells were fixed, permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience; San Diego, CA), and stained for expression of Foxp3 and/or CTLA-4. For all staining protocols, the buffer was 0.1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) buffer, and heat-inactivated normal human serum was used for blocking. Immunofluorescence flow cytometry data were acquired using the 4-laser LSRII flow cytometer (BD Biosciences, San Jose, CA) in the NJMS Flow Cytometry Core Facility. Data were analyzed using FlowJo software (Version 7.6.4; TreeStar, Stanford, CA). All gates for individual markers were set based on fluorescence minus 1 (FMO) control technique, in which cells are stained with all of the markers except 1 and gates are drawn based on fluorescence of the missing fluorochrome within its individual FMO tube.

Isolation of T cell subsets by fluorescence-activated cell sorting

For T_{reg} cell functional experiments, PBMC were stained with fluorochrome-conjugated CD4, CD25, and CD39 antibodies and separated into T_{reg} and T_{eff} populations by fluorescence-activated cell sorting (FACS) using the 4-laser FACSAria II cell sorter (BD Biosciences) in the NJMS Flow Cytometry Core Facility. Stringent gates were set using FMO, as described above, and $CD4^+$ $CD25^{hi}$ T_{regs} were <1.5% of $CD4^+$ T cells. The gating strategy for CD25 and sorting experiments is shown in Fig. 1. All cells were collected into 5-mL polypropylene tubes containing 40% fetal bovine serum in RPMI-1640 (Life Technologies, Grand Island, NY) complete medium supplemented with 1% penicillin–streptomycin–glutamine. The purity of the sorted cells was greater than 96%.

Suppression assay

$CD4^+CD25^-$ T_{effs} (4×10^4 cells/well) were stimulated with 0.5 µg plate-bound anti-CD3 plus irradiated allogeneic $CD3^-$ antigen-presenting cells (APCs; 1×10^5 cells/well)
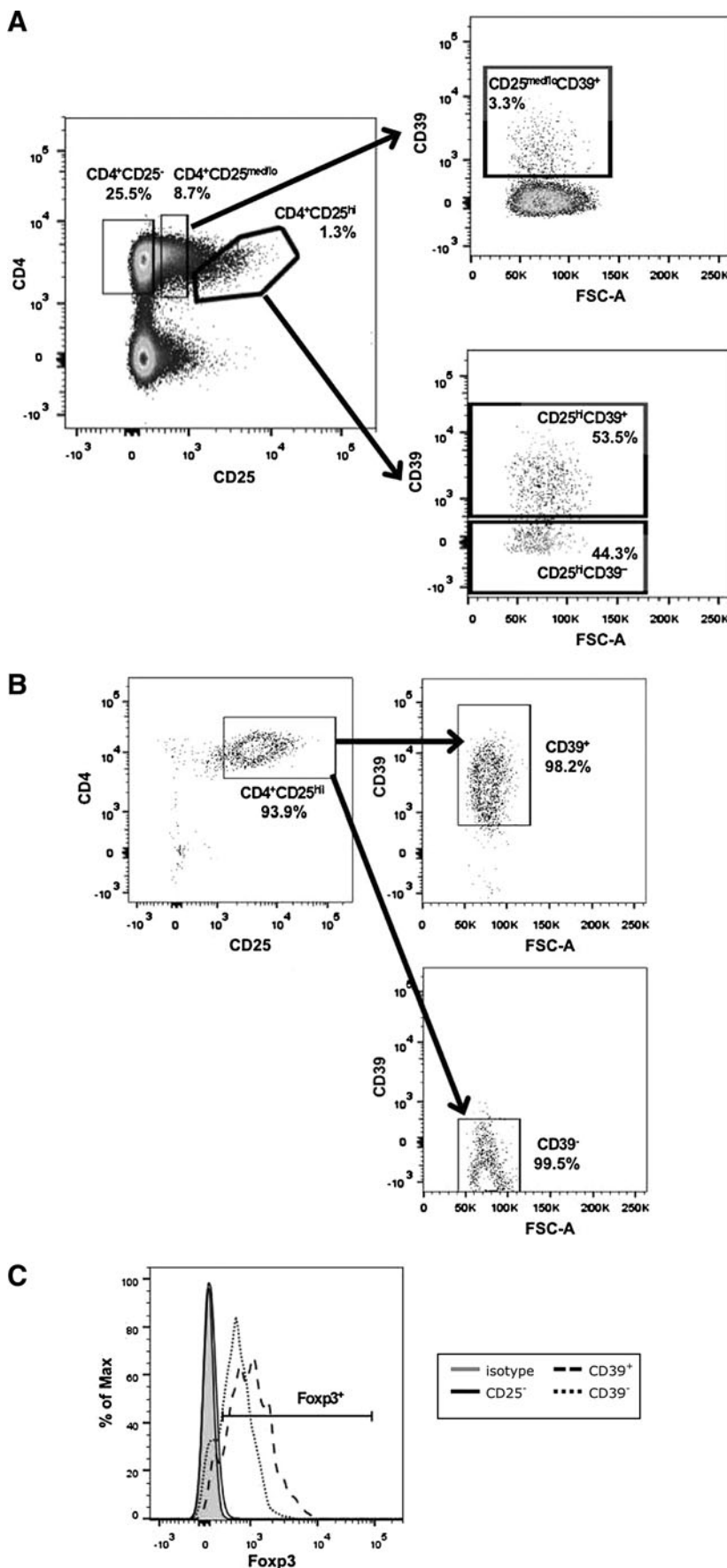
FIG. 1. Distinct populations of CD4$^+$CD25^{hi} T$_{regs}$ based on CD39 expression. PBMC were stained for surface expression of CD4, CD25, and CD39. **(A)** PBMC were first gated on lymphocytes using an FSC-A versus SSC-A dot plot, gated on singlets using an FSC-W versus FSC-H dot plot (not shown), gated on CD4$^+$CD25^{hi} or CD4$^+$CD25$^{med/lo}$ populations using a CD4 versus CD25 dot plot, and then gated on CD39$^+$ and CD39$^-$ populations using a CD39 versus FSC-A dot plot. **(B)** FACS was used to collect CD4$^+$CD25$^-$ T$_{effs}$, CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$, CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$, and CD4$^+$CD25$^{med/lo}$CD39$^+$ T cells. The purity of the sorted CD4$^+$CD25^{hi}CD39$^+$ and CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ from 1 representative donor is shown and **(C)** expression of Foxp3 in the sorted CD4$^+$CD25^{hi}CD39$^+$ and CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ is shown. FACS, fluorescence-activated cell sorting; FSC-A, forward scatter area; FSC-H, forward scatter height; FSC-W, forward scatter width; PBMC, peripheral blood mononuclear cells; SSC-A, side scatter area; T$_{regs}$, T regulatory cells.

and cocultured with or without sorted T_{regs} at a 1:1 ratio in 96-well round-bottomed plates for 6 days at 37°C in a humidified incubator with 5% CO_2 (Fletcher and others 2009). To ensure HLA MHC class II mismatch, $CD3^-$ cells were collected from the PBMC of 3 donors, by depletion of $CD3^+$ T cells by the Human CD3 MicroBeads kit (Miltenyi Biotec, Auburn, CA). The $CD3^-$ APCs from all 3 donors were mixed and irradiated at 20 Gy before culture. Proliferation was assessed after addition of thymidine (^{3}H-TdR; 1 μCi/well) for 18 h and measured using the RackBeta Liquid Scintillation counter (LKB Wallac, Turku, Finland). For experiments in which proliferation was not measured, cells were harvested on day 6 and stained for expression of various markers, including CD4, CD25, CD39, CD73, CTLA4, CCR6, IL-23R, and Foxp3.

Cytokine quantitation by enzyme-linked immunosorbent assay

IL-17 was measured by enzyme-linked immunosorbent assay (ELISA) using an anti-human IL-17 antibody (Clone No. 41809, 2,000 ng/mL; R&D Systems) as the capture antibody and a biotinylated polyclonal anti-human antibody (75 ng/mL; R&D Systems) as the detection antibody with quantification by reference to an rIL-17 standard (R&D Systems). IFN-γ was measured using a commercially available OptEIA kit (BD Pharmingen), according to the manufacturer's instructions. For all the ELISAs, 1% BSA in PBS was used as the blocking and dilution buffer. All samples were measured in duplicate wells, OD values were measured at an absorbance of 450 nm, and means of the duplicates were compared to a standard curve of known cytokine concentrations. The sensitivity of the ELISA was 7.8 pg/mL for IL-17 and 4.7 pg/mL for IFN-γ.

Intracellular staining for Stat phosphorylation

Phosphorylation of Stat3 and Stat1 was analyzed using intracellular staining following 6 days of activation of FACS-sorted T cells as described (Purvis and others 2014). Cells were stained for surface expression of CD4 and CD25, fixed with 2% paraformaldehyde, permeabilized with 100% ice-cold methanol overnight, blocked with human serum, and stained with pStat antibodies (pY705;clone 4/P-Stat3 or pY701;clone4a/P-Stat1) both from BD Pharmingen for 1 h. Data were acquired using the LSRII flow cytometer and analyzed using FlowJo software, as described above.

Stat3 inhibition

$CD4^+CD25^-$ T_{effs} and $CD4^+CD25^{hi}$ T cells with or without CD39 expression were isolated by FACS, as described above. FACS-sorted $CD4^+$ T cells and T_{regs} were then incubated at 37°C for 16 h in the absence or presence of Stattic V, a small molecule inhibitor of Stat3 activation and dimerization (50 ng/mL; Sigma-Aldrich). Following pretreatment with Stattic V, treated and untreated $CD4^+CD25^-$ T_{effs}, $CD4^+CD25^{hi}CD39^+$ T_{regs}, and $CD4^+CD25^{hi}CD39^-$ T cells (4×10^4 cells/well) were cultured in anti-CD3-coated wells in the presence of irradiated allogeneic $CD3^-$ APCs (1×10^5/well). Untreated $CD4^+CD25^-$ T_{effs} were also cultured with treated and untreated $CD4^+CD25^{hi}CD39^+$ T_{reg} cells and $CD4^+CD25^{hi}CD39^-$ T cells at a 1:1 ratio. After 5 days, cells were harvested and stained for analysis of Stat3

and Stat1 phosphorylation by flow cytometry, and supernatants were collected for analysis of IFN-γ and IL-17 production by ELISA.

Statistical analysis

Data were analyzed by looking at mean differences in fluorescence, cytokine levels and suppression of proliferation between the different cell populations or culture conditions using a repeated measures 1-way ANOVA (F-test) with a 1-tailed alpha level of 0.05 or Student's t-test, as appropriate, using Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA). Tukey's posttest was used to compare pairs of data sets following ANOVA. All experiments were analyzed utilizing appropriate statistical tests for parametric and nonparametric analyses on consultation with the biostatistics core facility at NJMS.

Results

$CD39^+$ human T_{regs} suppress T_{eff} proliferation, IFN-γ, and IL-17 production

To access the functional role of CD39 on T_{regs}, we examined the ability of FACS-sorted $CD4^+CD25^{hi}CD39^+$ and $CD4^+CD25^{hi}CD39^-$ T_{regs} to suppress proliferation and cytokine production of $CD4^+CD25^-$ T_{effs}. Human $CD4^+$ T cells were sorted into highly purified $CD4^+CD25^-$, $CD4^+CD25^{hi}CD39^+$, $CD4^+CD25^{hi}CD39^-$, and $CD4^+CD25^{med/lo}CD39^+$ T cell populations, using the gating strategy shown in Fig. 1A. Purity of the sorted populations was >98% (Fig. 1B). Foxp3 expression was higher in the $CD4^+CD25^{hi}CD39^+$ compared to the $CD4^+CD25^{hi}CD39^-$ T cells (Fig. 1C). $CD4^+CD25^{hi}CD39^+$ T cells suppressed the proliferation of $CD4^+CD25^-$ T_{effs} (mean ± standard error of the mean, 74% ± 12%), but $CD4^+CD25^{hi}CD39^-$ T cells were not capable of suppressing this response (Fig. 2A). To determine whether expression of CD39 or high CD25 expression was critical for the suppressive ability of these T_{regs}, we examined the ability of $CD4^+CD25^{med/lo}CD39^+$ T cells to inhibit the proliferation of $CD4^+CD25^-$ T_{effs}. Similar to the $CD4^+CD25^{hi}CD39^-$ T cells, $CD4^+CD25^{med/lo}CD39^+$ T cells were unable to suppress proliferation of $CD4^+CD25^-$ T_{effs} (Fig. 2B).

We next examined the ability of the $CD4^+CD25^{hi}CD39^+$ and $CD4^+CD25^{hi}CD39^-$ T cell subsets to inhibit cytokine production, as it has been demonstrated that proliferation and cytokine production are separate functions (Sojka and Fowell 2011). IFN-γ production by $CD4^+CD25^-$ T_{effs} was inhibited both by the $CD4^+CD25^{hi}CD39^+$ and $CD4^+CD25^{hi}CD39^-$ T cells ($P < 0.001$ and $P < 0.01$, respectively), with slightly stronger inhibition by the $CD39^+$ T_{reg} cells (Fig. 2C). Moreover, the $CD4^+CD25^{hi}CD39^+$ T cells significantly inhibited IL-17 production by $CD4^+CD25^-$ T_{effs} ($P < 0.05$), whereas the $CD4^+CD25^{hi}CD39^-$ T cells did not inhibit IL-17 production (Fig. 2C). Thus, CD39 expression on $CD4^+CD25^{hi}$ T cells denotes a subset of T_{regs} that suppress IL-17 production, while inhibiting T cell proliferation and IFN-γ production, confirming a previous report (Fletcher and others 2009). In contrast, the $CD4^+CD25^{hi}CD39^-$ T cells have regulatory activity that is restricted to inhibition of IFN-γ production.

To compare the ability of the CD25-expressing $CD4^+$ T_{regs} to secrete cytokines, we measured cytokine levels produced by $CD4^+CD25^{hi}CD39^+$, $CD4^+CD25^{med/lo}CD39^+$, and $CD4^+$
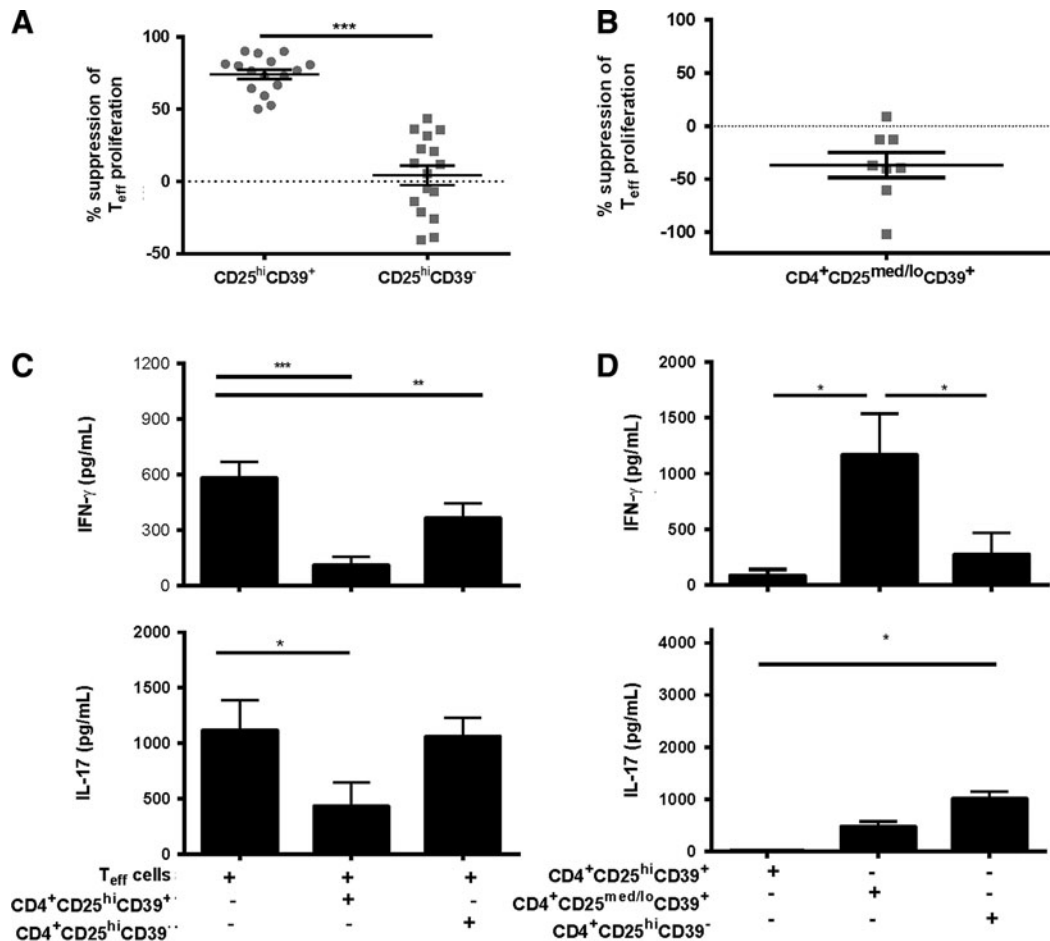
FIG. 2. CD39$^+$ T$_{regs}$ suppress proliferation, IFN-γ production, and IL-17 production of CD4$^+$CD25$^-$ T$_{effs}$. CD4$^+$CD25$^-$ T$_{eff}$ cells, CD4$^+$CD25^{hi}CD39$^+$ T$_{reg}$ cells, CD4$^+$CD25^{hi}CD39$^-$ T$_{reg}$ cells, and CD4$^+$CD25$^{med/lo}$CD39$^+$ T cells were collected by FACS. **(A)** CD4$^+$CD25$^-$ T$_{effs}$ were stimulated with plate-bound anti-CD3 plus irradiated allogeneic CD3$^-$ APCs and cultured alone or with sorted CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ or CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ at a 1:1 ratio for 6 days in 96-well round-bottomed plates. Proliferation was determined by measuring tritiated thymidine (^{3}H-TdR) incorporation during the final 18 h of the assay. Percent suppression was calculated using the formula [1 $-$ (co-culture cpm/T$_{eff}$ alone cpm)] $\times$ 100. **(B)** CD4$^+$CD25$^-$ T$_{effs}$ were stimulated as in **(A)** and cultured alone or with CD4$^+$CD25$^{med/lo}$CD39$^+$ T cells at a 1:1 ratio for 6 days and proliferation was measured by the amount of radiolabel uptake. **(C)** Supernatants from cultures in **(A)** were collected on day 5 and IFN-γ and IL-17 were measured by ELISA. **(D)** CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$, CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$, and CD4$^+$CD25$^{med/lo}$CD39$^+$ T cells were cultured alone or with plate-bound anti-CD3, and irradiated allogeneic APCs and supernatants were collected on day 5 and levels of IFN-γ and IL-17 were measured by ELISA. Data are expressed as the mean $\pm$ SEM. For **(A)** $n = 16$ different donors, **(B)** $n = 8$ different donors, **(C, D)** $n = 9$ different donors. Statistical analysis was performed using repeated measures ANOVA with Tukey's *post hoc* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. APCs, antigen-presenting cells; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean.

CD25^{hi}CD39$^-$ T cells on activation. The CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ produced very low levels of IFN-γ and undetectable amounts of IL-17 (Fig. 2D). The CD4$^+$CD25^{hi}CD39$^-$ T cell subset produced higher (not significant) amounts of IFN-γ and significantly higher levels of IL-17 on activation ($P < 0.05$) compared to the CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$. The nonregulatory CD4$^+$CD25$^{med/lo}$CD39$^+$ T cells produced significantly higher levels of IFN-γ ($P < 0.05$) and slightly but not significantly higher levels of IL-17 compared to the CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ (Fig. 2D).

Phenotypic analysis of CD39$^+$ human T$_{regs}$

The finding that human CD4$^+$CD25^{hi}CD39$^+$ and CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ were functionally different prompted us to perform a phenotypic analysis on these regulatory subsets. CD39 expression was significantly higher on the CD4$^+$CD25^{hi} T$_{regs}$ (44%$\pm$17%) in comparison to CD4$^+$CD25$^-$ (2.8%$\pm$1%; $P < 0.001$) and CD4$^+$CD25$^{med/lo}$ (9%$\pm$4%; $P < 0.001$) T cells (Fig. 3A, B). CD39 expression on CD4$^+$CD25^{hi} T$_{regs}$ was highly variable between donors, with expression ranging from 15% to 76% (Fig. 3B). Analysis of the T$_{reg}$-specific transcription factor Foxp3 revealed that CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ (76%$\pm$16%) expressed significantly higher levels ($P < 0.001$) compared to CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ (56%$\pm$17%) (Fig. 3C). CD73 surface coexpression with CD39 was very low in CD4$^+$CD25^{hi} T$_{regs}$ ranging from below 1% to 10% (Fig. 3D). CD4$^+$CD25$^+$ T cells include both CD4$^+$CD25^{hi} and CD4$^+$CD25$^{med/lo}$ T cells. CD73 was expressed without CD39 on a minor
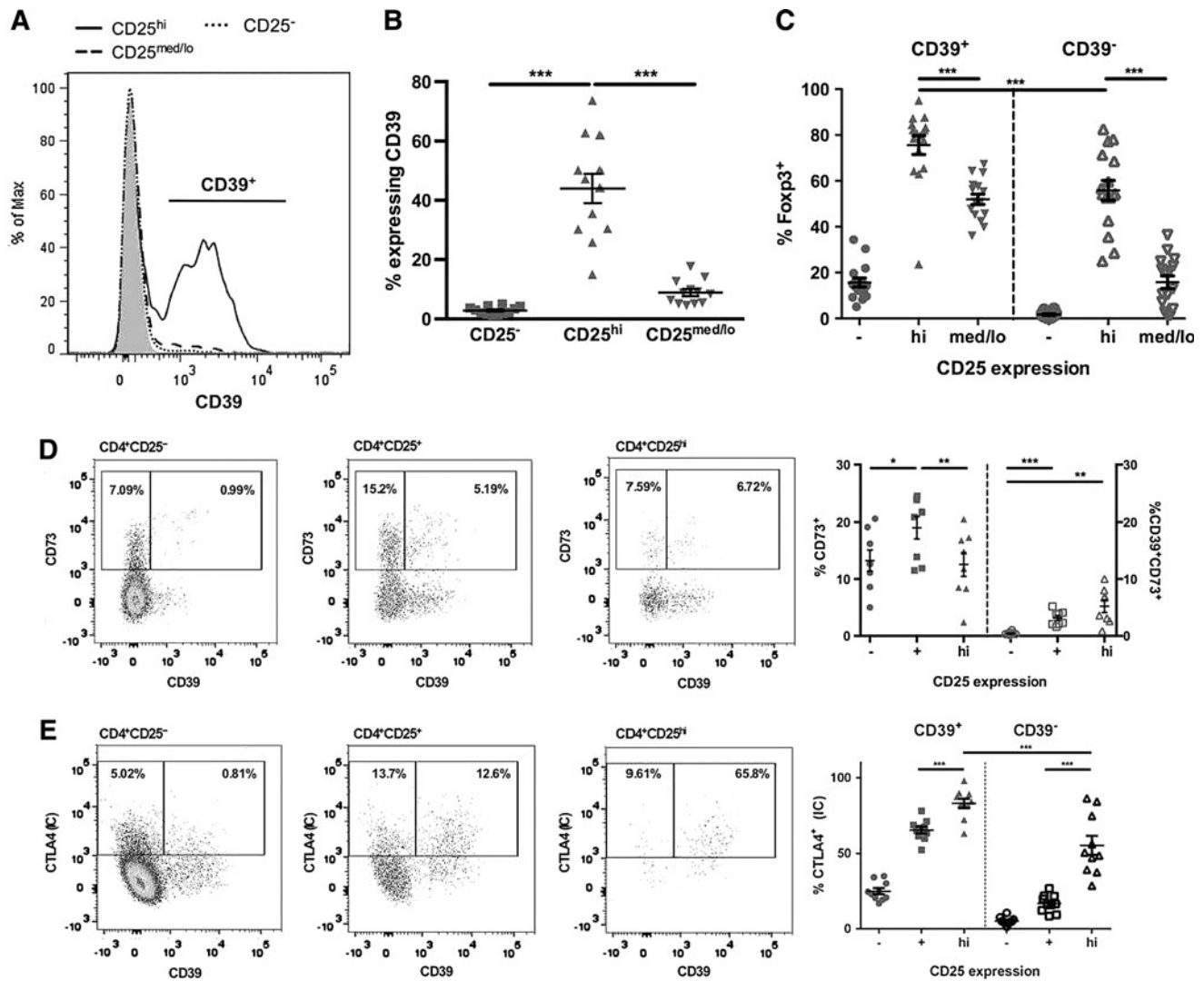
FIG. 3. CD39 is expressed on human CD4$^+$ T$_{regs}$ with high Foxp3 expression. Resting, *ex vivo* PBMC were analyzed for expression of surface CD25, CD39, CD73, and intracellular CTLA4 and Foxp3. **(A, B)** Cells were stained for surface expression of CD4, CD25, and CD39. **(C)** Cells were permeabilized with the Foxp3/Transcription Factor Staining Buffer Set and stained for intracellular Foxp3 expression. Dot plots show intracellular expression of Foxp3 on CD39$^+$ versus CD39$^-$ subsets of CD4$^+$CD25$^-$, CD4$^+$CD25^{hi}, and CD4$^+$CD25$^{med/lo}$ T cell populations. **(D)** Cells were stained for surface expression of CD4, CD25, CD39, and CD73. Quadrant analyses show coexpression of surface CD39 and CD73 on CD4$^+$CD25$^-$, CD4$^+$CD25$^+$, and CD4$^+$CD25^{hi} T cell populations. CD4$^+$CD25$^+$ T cells include both CD4$^+$CD25^{hi} and CD4$^+$CD25$^{med/lo}$ T cells. Dot plots show surface expression of CD73 and coexpression of CD39 and CD73 on CD4$^+$CD25$^-$, CD4$^+$CD25$^+$, and CD4$^+$CD25^{hi} T cell populations. **(E)** Cells were stained for surface expression of CD4, CD25, CD39, and intracellular CTLA4. Quadrant analyses show coexpression of surface CD39 and intracellular CTLA4 on CD4$^+$CD25$^-$, CD4$^+$CD25$^+$, and CD4$^+$CD25^{hi} T cell populations. Dot plots show intracellular expression of CTLA4 on CD39$^+$ versus CD39$^-$ subsets of CD4$^+$CD25$^-$, CD4$^+$CD25$^+$, and CD4$^+$CD25^{hi} T cell populations. For **(A)** data from 1 representative experiment are shown in histogram. *Gray* area is FMO control; for **(B, C)** $n = 12$ different donors, for **(D, E)** data from 1 representative experiment are shown in quadrant analyses and $n = 8$ **(D)** and $n = 9$ **(E)** different donors. Data are expressed as the mean $\pm$ SEM. Statistical analysis was performed using repeated measures ANOVA with Tukey's *post hoc* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. FMO, fluorescence minus 1.

fraction of CD25^{hi} T$_{regs}$. We observed very low levels of intracellular CD73 in the different T cell subsets, with almost no coexpression of CD39 and intracellular CD73 (data not shown). The CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ have a significantly higher ($P < 0.05$) percentage of CD27$^+$ CD127$^-$ cells (82.0% $\pm$ 4.3%) than the CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ (66.1% $\pm$ 8.4%). CD45RO was expressed on almost all of the CD4$^+$CD25^{hi}CD39$^+$ T$_{reg}$ cells and CD4$^+$CD25^{hi}CD39$^-$ T$_{reg}$ cells (data not shown).

As CTLA-4 is thought to be important for T$_{reg}$ cell function, we investigated the expression of surface and intracellular CTLA-4 on *ex vivo* isolated PBMC. CD4$^+$CD25^{hi} T$_{regs}$ have low (7.3 $\pm$ 2.4), although significantly higher, surface CTLA-4 expression than CD4$^+$CD25$^-$ (0.03 $\pm$ 0.1; $P < 0.04$) T cells. CD4$^+$CD25^{hi}CD39$^+$ T$_{regs}$ expressed significantly more intracellular CTLA-4 (83% $\pm$ 10%) compared to CD4$^+$CD25^{hi}CD39$^-$ T$_{regs}$ (55% $\pm$ 20%; $P < 0.001$) (Fig. 3E). The phenotyping data indicate that CD39$^+$ T$_{regs}$

have a higher expression of Foxp3 and intracellular CTLA-4 but similar expression of CD45RO to CD39$^-$ T_{regs}.

CCR6 and IL-23R are expressed on the surface of suppressive CD39$^+$ T_{reg} cells following activation

Evidence from studies on murine T_{regs} shows that upregulation of chemokine receptors is critical for the ability of T_{regs} to suppress specific types of immune responses. In addition, 1 study classified human Foxp3$^+$ human T_{regs} as Th1-like, Th2-like, and Th17-like T_{reg} subsets based on their expression of subset-associated chemokine receptors and transcription factors (Duhen and others 2012). Since CD39$^+$ T_{regs} suppress *in vitro* IL-17 secretion, we next investigated the expression of CCR6 and IL-23R on CD4$^+$CD25^{hi}CD39$^+$ and CD4$^+$CD25^{hi}CD39$^-$ T_{regs}. CCR6 expression was very low on resting, *ex vivo* human CD4$^+$CD25^{hi} T_{regs} (2.9% ± 1.3%) and CD4$^+$CD25$^-$ T cells (1.6% ± 0.5%). CD39$^+$ T_{regs} markedly upregulated CCR6 expression following activation ($P < 0.001$)

in comparison to the CD39$^-$ T_{reg} cells (Fig. 4A, D). Furthermore, CD39$^+$ T_{regs} strongly upregulated IL-23R expression on activation ($P < 0.01$) (Fig. 4B, E). CCR6 and IL-23R were coexpressed on significantly more CD39$^+$ T_{regs} then CD39$^-$ T_{regs} ($P < 0.001$) (Fig. 4C, F). CD39$^-$ T_{regs} and CD4$^+$ T_{effs} expressed low levels of either CCR6 or IL-23R or their coexpression following activation (Fig. 4D–F). These findings show that the CD39$^+$ T_{regs} that suppress IL-17 production express the Th17-associated surface markers CCR6 and IL-23R.

Stat3 phosphorylation contributes to CD39$^+$ T_{reg}-mediated suppression of IL-17

As T_{regs} may be specialized to suppress a specific CD4$^+$ T cell subset by expressing the hallmark transcription factor of that specific Th subset (Chaudhry and others 2009; Koch and others 2009; Zheng and others 2009), we analyzed phosphorylation of the Th17- and Th1-associated transcription factors Stat3 and Stat1, in CD4$^+$ T_{effs} and CD39$^+$ T_{regs}
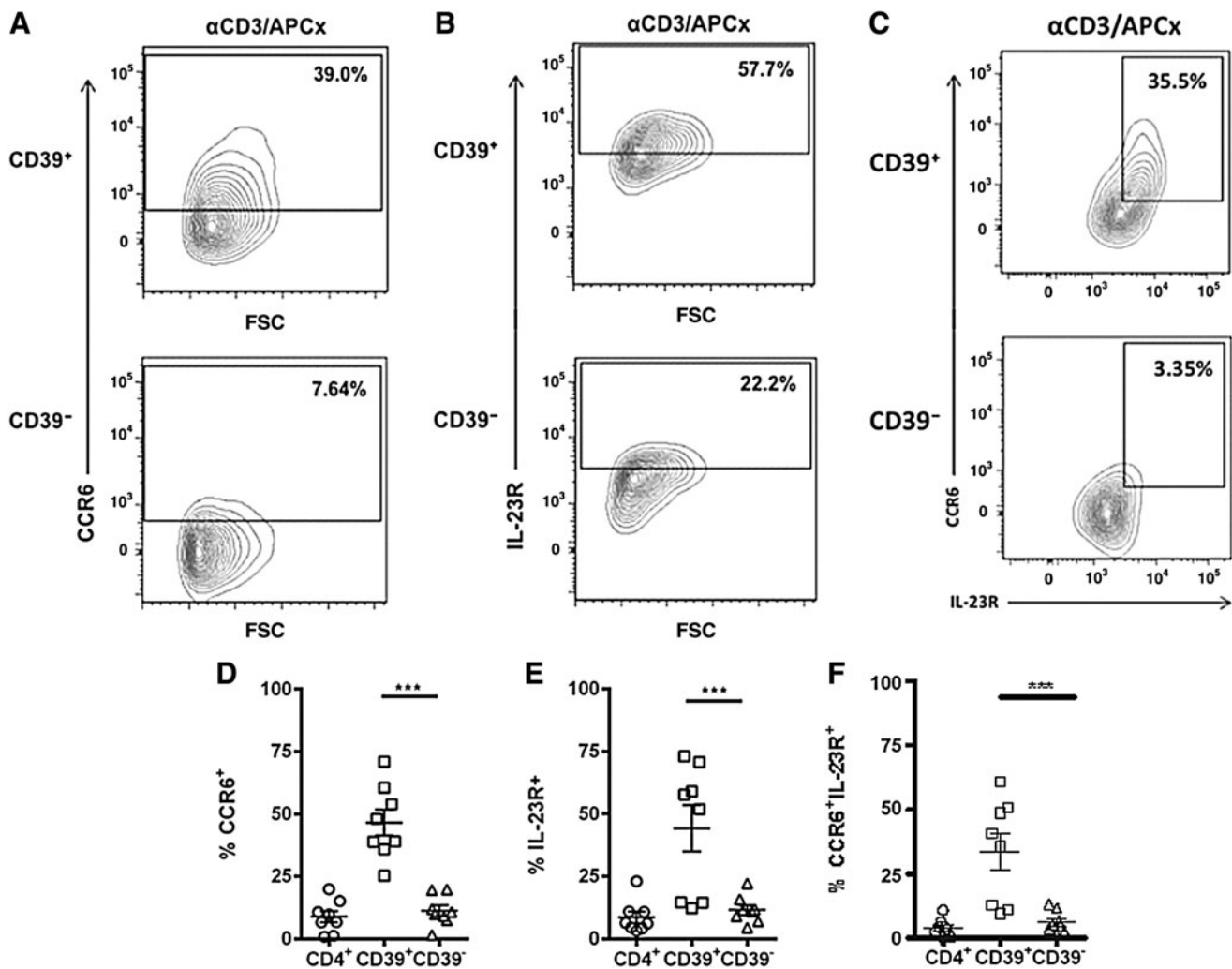

FIG. 4. Human CD39$^+$ T_{regs} express CCR6 and IL-23R following activation. CD4$^+$CD25$^-$ T_{effs}, CD39$^+$ T_{regs}, and CD39$^-$ T_{regs} were collected by FACS and cultured alone in the presence of plate-bound anti-CD3 and irradiated allogeneic APCs for 6 days. Following activation, cells were stained with fluorochrome-conjugated antibodies to CD4, CD25, CD39, CCR6, and IL-23R, and flow cytometry was used to analyze surface expression of CCR6 (**A, D**), IL-23R (**B, E**), and CCR6 and IL-23R coexpression (**C, F**) on activated CD4$^+$CD25$^-$ T_{effs}, CD4$^+$CD25^{hi}CD39$^+$ T_{regs}, and CD4$^+$CD25^{hi}CD39$^-$ T_{regs}. Contour plots in (**A–C**) show 1 representative donor out of 8 different donors. Data are expressed as the mean ± SEM. Statistical analysis was performed using repeated measures 1-way ANOVA with Tukey's *post hoc* test. ***$P < 0.001$.
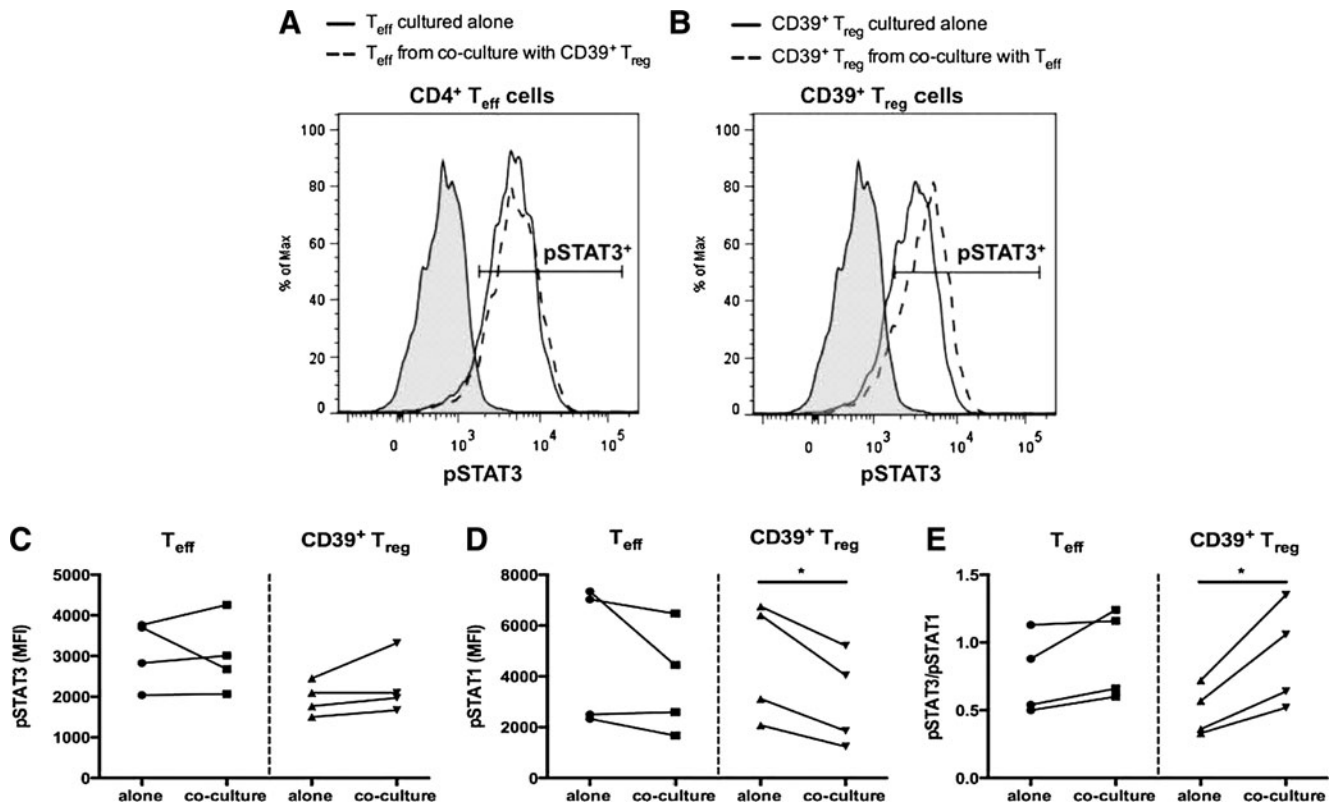
FIG. 5. Levels of Stat3 and Stat1 phosphorylation in $CD39^+$ T_{regs}. $CD4^+CD25^-$ T_{effs} and $CD4^+CD25^{hi}CD39^+$ T_{regs} were collected by FACS and cultured alone or in $T_{eff}:T_{reg}$ cocultures at a 1:1 ratio for 6 days in the presence of plate-bound anti-CD3 and irradiated allogeneic APCs. **(A–E)** Following 6 days of activation, cells were harvested and stained for surface expression of CD3, CD4, and CD25 as well as intracellular expression of pStat3 and pStat1 as described (Purvis and others 2014). Expression of pStat3 and pStat1, as well as the pStat3/pStat1 ratio, was determined using flow cytometric analysis of T_{effs} and T_{regs} collected from alone and coculture conditions. Histograms in **(A, B)** show data from 1 representative donor. *Gray* area is FMO control. MFI of Stat1 and Stat3 for 4 donors from 3 independent experiments is shown in **(C–E)**. Statistical analysis was performed using paired Student's *t*-test **(C–E)** *$P < 0.05$. FMO, fluorescence minus 1; MFI, mean fluorescence intensity.

activated alone and when cocultured together. $CD4^+$ T_{effs} expressed similar levels of pStat3 and pStat1 regardless of whether they were activated alone or in coculture with $CD39^+$ T_{regs} (Fig. 5A, C, and D). $CD39^+$ T_{regs} activated alone phosphorylated Stat3, and slightly higher levels of pStat3 were seen following coculture with $CD4^+$ T_{effs}. $CD39^+$ T_{regs} activated alone phosphorylated Stat1, and significantly lower levels of pStat1 were seen following coculture with $CD4^+$ T_{effs} (Fig. 5B–D). Analysis of the ratio of pStat3/pStat1 showed a significant ($P < 0.05$) preference toward Stat3 phosphorylation in the $CD39^+$ T_{regs} following coculture with $CD4^+$ T_{effs}, whereas the pStat3/pStat1 ratio did not significantly differ in the T_{effs} regardless of whether these cells were cultured alone or in coculture with $CD39^+$ T_{regs} (Fig. 5E). These data suggest that the relative levels of pStat3 and pStat1 may contribute to the functional suppression mediated by $CD39^+$ T_{regs}.

As Stat3 is associated with IL-17 production, we investigated the role of Stat3 activation in the production and suppression of IL-17. Sorted $CD4^+$ $CD25^-$ T_{effs}, $CD39^+$ T_{regs}, and $CD39^-$ T_{regs} were pretreated with the Stat3 inhibitor Stattic V for 16 h before activation. Stattic-treated and untreated $CD39^+$ and $CD39^-$ T_{regs} were cultured alone and in 1:1 cocultures with untreated $CD4^+$ $CD25^-$ T_{effs} and activated with plate-bound anti-CD3 and irradiated allogeneic APCs. In the absence of Stattic treatment, $CD4^+CD25^{hi}CD39^+$ T cells significantly ($P < 0.02$) suppressed IL-17 production of $CD4^+CD25^-$ T_{effs}, whereas $CD4^+CD25^{hi}CD39^-$ T cells

slightly enhanced although not significantly IL-17 production when cultured with $CD4^+CD25^-$ T_{effs} (Fig. 6A). IL-17 production by $CD4^+$ T_{effs} cultured in the absence of T_{regs} was significantly inhibited ($P < 0.01$) by Stattic pretreatment (Fig. 6A). Likewise, production of IL-17 by the $CD39^-$ T_{regs} activated in the absence of $CD4^+CD25^-$ T_{effs} was significantly ($P < 0.02$) inhibited by Stattic treatment (Fig. 6A). In contrast, inhibition of Stat3 activation in the $CD39^+$ T_{regs} resulted in a significant ($P < 0.02$) increase in IL-17 in cocultures of $CD4^+CD25^-$ T_{effs} and Stattic-pretreated $CD39^+$ T_{regs} (Fig. 6A, B). We also observed a slight but not significant decrease in IL-17 production in cocultures of $CD4^+CD25^-$ T_{effs} and Stattic-pretreated $CD39^-$ T_{regs} (Fig. 6A, B). The magnitude of reversal of IL-17 suppression by Stattic varied between donors, with the increase in IL-17 production ranging from 18% to 150%. These findings suggest that human $CD39^+$ T_{regs} inhibit IL-17 in a Stat3-dependent mechanism, as activation of Stat3 within these cells is required for suppression of IL-17.

Discussion

Human T_{regs} are a phenotypically and functionally heterogeneous population of $CD4^+$ T cells. Classifying diverse T_{reg} populations and understanding the mechanisms they use to regulate polarized immune responses are important for the development of disease-specific T_{reg}-based immunotherapy. Murine T_{regs} express canonical $CD4^+$ T effector
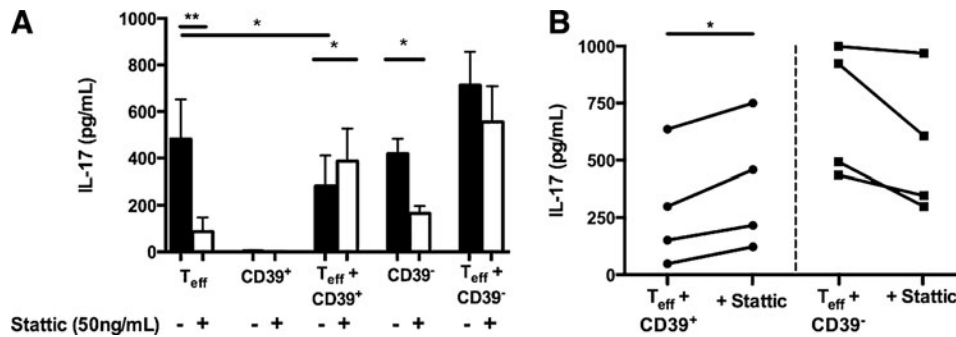
FIG. 6. Inhibition of Stat3 activation in the CD39$^+$ T_{regs} reverses their ability to suppress IL-17. CD4$^+$CD25$^-$ T_{effs}, CD4$^+$CD25^{hi}CD39$^+$ T_{regs}, and CD4$^+$CD25^{hi}CD39$^-$ T_{reg} cells were collected by FACS and incubated with the Stat3 inhibitor Stattic V (50 ng/mL) for 16 h at 37°C. Stattic-treated and untreated CD4$^+$CD25^{hi}CD39$^+$ T_{regs} and CD4$^+$CD25^{hi}CD39$^-$ T_{regs} were then cultured alone or at a 1:1 ratio with untreated CD4$^+$CD25$^-$ T_{effs} for 6 days in the presence of plate-bound anti-CD3 and irradiated allogeneic APCs. Stattic-treated and untreated CD4$^+$CD25$^-$ T_{effs} were cocultured alone under the same conditions. Supernatants were collected on day 6 and IL-17 production was measured by ELISA (**A, B**). *Black* bars represent Stattic untreated cultures, *white* bars represent Stattic treated cultures. Data represent the mean ± SEM for 4 different donors from 3 independent experiments. Statistical analysis was performed using repeated measures 1-way ANOVA with Tukey's *post hoc* test (**A**) or paired Student's *t*-test (**B**). *$P < 0.02$, **$P < 0.01$.

cell-associated transcription factors to control polarized Th1-, Th2-, and Th17-driven immune responses (Chaudhry and others 2009; Koch and others 2009; Zheng and others 2009). In this study, we examined the molecular basis underlying the phenotypic and functional diversity of human T_{regs}. We demonstrate for the first time that human CD4$^+$CD25^{hi}CD39$^+$ T_{regs}, which suppress IL-17 production by CD4$^+$ T_{eff} cells, express the Th17-associated surface markers CCR6 and IL-23R. Moreover, phosphorylation of Stat3 and Stat1 by CD4$^+$CD25^{hi}CD39$^+$ T_{regs} contributes to their suppressive effect. Suppression of IL-17 by CD4$^+$CD25^{hi}CD39$^+$ T_{regs} occurs via a Stat3-dependent mechanism as inhibition of Stat3 activation in the CD39$^+$ T_{regs} reverses their ability to suppress IL-17. CD4$^+$CD25^{hi}CD39$^-$ T_{regs} are not endowed with the ability to inhibit IL-17, as they do not upregulate CCR6 or IL-23R, and furthermore, they secrete IL-17. Our study suggests that CD4$^+$CD25^{hi}CD39$^+$CCR6$^+$IL-23R$^+$Foxp3$^+$ T_{regs} represent human Th17-specific regulatory T cells (T_{reg}17).

In searching for "signature" T_{reg} markers, we observed that expression of the ectonucleotidase CD39 was largely limited to the CD25^{hi} subset of CD4$^+$ T cells, with highly variable expression between donors, as previously reported (Fletcher and others 2009; Dwyer and others 2010). In contrast, nearly all CD4$^+$CD25^{hi}Foxp3$^+$ T_{reg} cells were reported positive for CD39 (Mandapathil and others 2010). We found that CD4$^+$CD25^{hi}CD39$^+$ T_{regs} expressed the highest levels of intracellular Foxp3 and CTLA-4 in comparison to CD4$^+$CD25^{hi}CD39$^-$ T_{regs}. Only a small percentage of CD25^{hi} T_{regs} coexpressed surface CD39 and CD73, in agreement with other human studies (Borsellino and others 2007; Dwyer and others 2010; Rissiek and others 2015).

To assess the suppressive capacity of these T_{reg} populations, we used FACS-sorted CD4$^+$ T_{effs} and T_{regs} activated with plate-bound CD3 and irradiated allogeneic APC (Borsellino and others 2007; Fletcher and others 2009; Miyara and others 2009; Duhen and others 2012). We observed that CD4$^+$CD25^{hi}CD39$^+$ and CD4$^+$CD25^{hi}CD39$^-$ T cells have distinct suppressive capabilities. CD4$^+$CD25^{hi}CD39$^+$ T_{regs} have the ability to suppress the proliferative response, as well as IL-17 and IFN-γ production by CD4$^+$ T_{effs}, whereas CD4$^+$CD25^{hi}CD39$^-$ T_{regs} only suppress IFN-γ production by CD4$^+$ T_{effs}. These results

suggest that T_{reg} cell subsets use different mechanisms for suppressing T_{eff} proliferation and cytokine production, as has been reported in the murine system (Sojka and Fowell 2011). One study showed that both CD4$^+$CD25^{hi}CD39$^+$ and CD4$^+$CD25^{hi}CD39$^-$ T_{regs} suppressed proliferation and IFN-γ production and that the CD4$^+$CD25^{hi}CD39$^+$ T_{regs} inhibited IL-17 production (Fletcher and others 2009).

The concept of lineage-specific T_{regs} is supported by studies demonstrating that mouse T_{regs} expressing T-bet inhibit Th1 cells, IRF4-expressing T_{regs} inhibit Th2 cell function, and pStat3-expressing T_{regs} inhibit Th17-mediated inflammation (Chaudhry and others 2009; Koch and others 2009; Zheng and others 2009). Whether specialization and differentiation of human T_{regs} occur has not been shown and is important to determine since differences exist between mouse and human T_{regs} regarding subset specificities and expression of markers. We have demonstrated that expression of CCR6 and IL-23R was specifically upregulated on CD39$^+$ T_{regs} following activation. These data suggest that CD4$^+$CD25^{hi}CD39$^+$CCR6$^+$IL-23R$^+$ T_{regs} may be specifically primed to interact with Th17 cells, as they may migrate with Th17 cells to sites of inflammation. Murine models have shown that CCR6-expressing T_{regs} migrate with Th17 cells to sites of inflammation (Yamazaki and others 2008; Turner and others 2010). T_{regs} that lacked surface expression of CCR6 resulted in impaired renal trafficking in an animal model of glomerulonephritis (Kluger and others 2014). Furthermore, expression of IL-23R on these T_{regs} may allow for their expansion at sites of inflammation, as IL-23 is important for Th17 cell expansion and survival (Wilson and others 2007; Liu and Rohowsky-Kochan 2008). Likewise, expression of the IL-23R on the T_{regs} may deprive Th17 cells of essential survival factors. To our knowledge, this is the first report demonstrating that coexpression of CCR6 and IL-23R is upregulated on human T_{regs}.

In addition to specific expression of Th17 surface markers on the CD39$^+$ T_{regs}, our studies suggest that the relative levels of pStat3 and pStat1 may be important in inhibiting Th17 function. The increase in pStat3, in conjunction with the significant decrease in pStat1 in CD39$^+$ T_{regs} cocultured with CD4$^+$ T_{effs}, results in a significant increase in the pStat3 to pStat1 ratio. These data are in agreement with

studies of mouse T_{regs} that report increased pStat3 in IL-17-suppressing T_{regs} (Koch and others 2009; Kluger and others 2014). Further studies using small interfering RNA silencing of pStat3 and pStat1 are warranted to confirm the role of these factors in $CD39^+$ T_{reg} cell-mediated suppression. Moreover, it remains to be determined which Stat-inducing cytokines are involved. No IL-6 or IL-23 was detected in the activated $CD4^+$ T_{eff} and $CD39^+$ T_{reg} cocultures and slight increases in IL-10 were seen (data not shown).

The functional role of pStat3 in mediating the suppressive function of $CD39^+$ T_{regs} was demonstrated by inhibiting Stat3 activation in $CD39^+$ T_{regs}. A salient finding of our study is the demonstration that suppression of IL-17 by $CD4^+CD25^{hi}$ $CD39^+$ T_{regs} occurs via a Stat3-dependent mechanism, as inhibition of Stat3 activation in the $CD39^+$ T_{regs} reverses their ability to suppress IL-17. Treatment with a Stat3 inhibitor resulted in decreased IL-17 production by $CD4^+$ T_{effs} and by $CD4^+CD25^{hi}CD39^-$ T cells, whereas suppression of IL-17 production by $CD39^+$ T_{regs} was significantly reversed on treatment of T_{regs} with the Stat3 inhibitor. Hence, Stat3 activation is involved in IL-17 production as well as in suppressing IL-17 by T_{regs}. Our findings are in agreement with murine data describing that lineage-specific $T_{reg}17$ cells expressed pStat3 for T_{reg} cell suppression of IL-17 production (Chaudhry and others 2011; Kluger and others 2014, 2016). CCR6 expression on human T_{regs} also appears dependent on Stat3, as patients with dominant-negative Stat3 mutations have greatly reduced CCR6 levels (Kluger and others 2014). Further studies are needed to characterize the mechanism by which activation of Stat3 in $CD39^+$ T_{regs} endows them with the ability to suppress Th17 responses. Unpublished data from our laboratory found that the immunosuppressive cytokine IL-35 does not appear to be involved.

Our studies differ from those of Goodman and others (2011), who reported that IL-6 inhibits T_{reg} cell function and that the IL-6-mediated loss of T_{reg} suppression requires phosphorylation of Stat3. They showed that the ability of T_{regs} to suppress proliferation of $CD4^+$ T_{effs} in response to CD3 and CD28 crosslinking is restored when Stat3 is inhibited. Only T_{reg}-mediated suppression of the proliferative response was examined. We specifically studied $CD39^+$ T_{regs} and their ability to inhibit IL-17 production by T_{eff} cells stimulated with anti-CD3 and APCs. No exogenous IL-6 was added nor was any IL-6 detected in our culture system.

Distinct subsets of human $Foxp3^+$ human T_{regs} that phenotypically mirror effector Th cells were reported. Th1-like, Th2-like, and Th17-like T_{reg} subsets were identified based on their expression of subset-associated chemokine receptors and mRNA expression of the transcription factors T-bet, GATA-3, and $ROR\gamma t$, respectively (Duhen and others 2012). No differences in mRNA expression of IRF4 and Stat3 were seen among the different T_{reg} subsets. Although this study indicated a role for T_{reg} expression of a Th17-associated transcription factor, the specificity and function of these cells and mechanism of suppression remain to be elucidated.

As Th17 cells are implicated in various autoimmune diseases, Th17-specific $CD39^+$ T_{regs} may be utilized as treatment for these conditions. Due to the heterogeneity of this population, T_{reg} cell transplantation has not yet been effective. We have shown that while $CD4^+CD25^{hi}CD39^+$ T_{regs} suppressed T_{eff} proliferation and production of IL-17, the $CD4^+CD25^{hi}$ T cell subset that lacks CD39 expression proliferated and produced IL-17. If $CD4^+CD25^{hi}$ T_{regs} were used to treat a Th17-skewed autoimmune disease such as

multiple sclerosis (MS), production of IL-17 by $CD4^+CD25^{hi}CD39^-$ T_{regs} may counteract the beneficial effects of the $CD39^+$ T_{regs}. Thus, since T_{regs} display a degree of plasticity, it is crucial to understand the complex interplay of the transcription factors in controlling T_{reg} suppressive versus pathogenic function. It will be important to further investigate whether the impaired suppressive activity of $CD4^+CD25^{hi}CD39^+$ T_{regs} in MS patients is associated with a dysfunction in Stat3 (Fletcher and others 2009). In the future, targeting of T helper cell subset-specific T_{regs} may be utilized to reduce specific types of inflammation in humans.

Author Disclosure Statement

No competing financial interests exist.

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