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IL-7 Modulates In Vitro and In Vivo Human Memory T Regulatory Cell Functions through the CD39/ATP Axis

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The heterogeneity of human regulatory T cells (Tregs) may explain the discrepancies between studies on Tregs in physiology and pathology. Contrasting effects of IL-7 on the expansion and survival of human Tregs were reported. Therefore, we investigated the effects of IL-7 on the phenotype and function of well-characterized populations of human Tregs. We show that IL-7 signals via the CD127 receptor on naive, memory, and activated memory Tregs sorted from the blood of healthy donors, but it does not affect their proliferation. In contrast, IL-7 affects their suppressive capacities differently. This effect was modest on naive Tregs but was dramatic (90%) on memory Tregs. We provide evidence that IL-7 exerts a synergistic effect through downmodulation of the ectoenzyme CD39, which converts ATP to ADP/AMP, and an increase in ATP receptor P2X7. Both effects lead to an increase in the ATP-mediated effect, tipping the balance to favor Th17 conversion. Using an IL-7 therapeutic study, we show that IL-7 exerts the same effects in vitro and in vivo in HIV-infected individuals. Globally, our data show that IL-7 negatively regulates Tregs and contributes to increase the number of tools that may affect Treg function in pathology. ***The Journal of Immunology*, 2013, 191: 3161–3168.**

$CD4^+$ regulatory T cells (Tregs) mediate dominant and long-lasting tolerance. Recent studies showed that the phenotype and suppressive capacity of Tregs are highly heterogeneous. Human Tregs are described based on CD4, Foxp3, and high CD25 expression. In 2009, Miyara et al. (1) divided $CD4^+$ $Foxp3^+CD25^+$ cells into various subpopulations: resting or naive Tregs (nTregs; $CD45RA^+Foxp3^{++}CD25^{++}$), activated memory or effector Tregs ($CD45RA^-Foxp3^{high}CD25^{high}$), and memory Tregs ($CD45RA^-Foxp3^{++}CD25^{++}$). Moreover, it was shown that expression of CD127, the α-chain of the IL-7R, allows the distinction between CD127(low) Tregs and CD127(high) conventional T cells (2).

Different pathways and molecules involved in the suppression of immune activation are important to provide insight into the control of peripheral tolerance and to identify important therapeutic targets. Tregs may interfere directly with T effector function through several mechanisms. We (3) and other investigators (4, 5) recently described the involvement of CD39 in Treg function. CD39 is an ectoenzyme that, together with CD73, hydrolyzes extracellular pools of ATP into ADP and/or AMP to adenosine. Adenosine inhibits T cell proliferation and IL-2 production through adenosine receptor A2AR by stimulating the generation of cAMP. We reported that CD39 gene polymorphism is associated with a slower progression to AIDS, revealing this molecule as an important player in the suppression of anti-HIV responses (3).

Manipulation of Treg functions is an interesting therapeutic approach in numerous clinical settings. The use of cytokines may represent one way to modulate Treg function. The failure of IL-2 therapy during HIV infection has been linked to its ability to induce the proliferation of Tregs (6, 7), a property that has been used to improve hepatitis C virus–induced vasculitis (8). Recently, the role of other γc cytokines in Treg function and survival was investigated (9–19). Because Tregs are known to harbor low expression of CD127 (2), the role of this cytokine in Treg homeostasis has been ignored for a long time. However, several reports in mice and humans showed that IL-7 may impact the function and survival of Treg populations (9–19). However, precise mechanisms of action of IL-7 on Treg functions are still unclear.

One other important question regarding Tregs concerns their plasticity. Although they have been considered to represent stable populations, recent observations challenged this notion, suggesting that, under inflammatory conditions, they may acquire effector functions (20). Th17 $CD4^+$ T cells, which express the transcription factor RORγt, belong to the third effector lineage of $CD4^+$ T cells that develops independently of the classical Th1 and Th2 programs. Th17 cells and Tregs use reciprocal developmental pathways involving TGF-β (21). Although both cell types could compete for their differentiation from uncommitted T cells, recent reports showed that environmental factors could reverse Treg phenotype and function and redirect those cells toward a Th17 phenotype (22–25).

In this study, we investigated the role of IL-7 on the phenotype of Tregs and the expression of molecules involved in the suppressive

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Abbreviations used in this article: aTreg, activated regulatory T cell; c-ART, antiretroviral therapy; nTreg, naive regulatory T cell; PPAD, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid; Treg, regulatory T cell.

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functions of these cells. We found that IL-7 relieves the suppressive effect of nonnaive Tregs by modulating the expression of CD39. We showed an increased expression of RORγt and IL-17 mRNA and an increased production of IL-17. IL-7 also increased Treg expression of P2X7, a receptor of ATP, on activated Tregs (aTregs). We found that the Th17 switch of Tregs was blocked by antagonist of ATP receptor. Globally, our results show that IL-7 exerts two synergistic effects on Tregs by modulating CD39 and increasing the ATP receptor, leading to a switch of Tregs toward a Th17 phenotype. Moreover, we found that in vivo administration of IL-7 tipped the balance toward a higher expression of RORγt in HIV-infected patients.

Materials and Methods

Patient samples

Peripheral blood samples were collected from HIV$^-$ healthy donors seen at the *Centre Regional de Transfusion Sanguine* and from HIV-infected patients treated with antiretroviral therapy (c-ART) followed at Henri Mondor Hospital. Ethical committee approval and written informed consent from all subjects, in accordance with the Declaration of Helsinki, were obtained prior to study initiation. All HIV-infected patients were treated with c-ART, were virologically suppressed (viral load < 20 copies/ml), and had sustained CD4$^+$ T cell restoration (CD4$^+$ > 500/mm^3). Blood samples were also obtained from nine HIV-infected patients who were treated with IL-7 (26). These patients, who had CD4 counts between 101 and 400 cells/mm^3 and viral load < 50 copies/ml, received s.c. injections of rIL-7 on days 0, 7, and 14.

Cell sorting

Single-cell sorting was done with the MoFlo Legacy (Beckman Coulter, Marseille, France) to obtain various cell populations in Sterile FACS tubes (BD Falcon). CD4$^+$CD25$^+$ cells were isolated using an isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's guidelines. The purity of isolated cells was checked after separation by FACS analysis. CD4$^+$CD25$^+$ cells were then incubated or not with 10 ng/ml IL-7 (Cytheris) for 24 h in 48-well culture plates. Cells were washed, and three living Treg subpopulations (i.e., naive, activated, and memory Tregs) were defined on the basis of CD45RA/CD127/CD25 expression. CD8$^+$ cells were isolated from PBMCs by positive selection using CD8 Micro Beads (Miltenyi Biotec).

Flow cytometric analyses

The following mAbs were used in the study: CD3 allophycocyanin H7 (SK7; BD Pharmingen, San Diego, CA), Foxp3 FITC (PCH-101; eBioscience, San Diego, CA), CD4 Pacific Blue (RPA-T4; BD Pharmingen), CD4 FITC (SFCL12T4D11; Beckman Coulter), CD25 PE (4E3; Miltenyi Biotec), CD25 PC7 (B1.49.9; Beckman Coulter), CTLA4 PE (AAPO02; R&D Systems, Minneapolis, MN), GITR allophycocyanin (110416; R&D Systems), CD8 PerCP (SK1; BD Biosciences, Le Pont de Claix, France), CD45RA PE Texas Red (2H4; Beckman Coulter), CD127 eFluor 710 (MB15-18C9; eBioscience), CD39 PE (TU66; BD Pharmingen), PDL1 PC7 (PDL1.3.1; Beckman Coulter), STAT-5 Alexa Fluor 488 (Py694; BD Biosciences), and Bcl-2 PE (100; BD Biosciences). The Live/Dead Fixable Aqua Dead Cell stain kit (Invitrogen, Carlsbad, CA) was used to exclude dead cells in all experiments.

Purified CD4$^+$CD25$^+$ cells were incubated or not with 10 ng/ml IL-7 for 24 h, and the expression of CTLA4, GITR, CD39, or PDL1 was analyzed on cell populations gated on CD45RA, CD127, FoxP3, and CD25 expression. Stained cells were analyzed on a 10-color LSR II flow cytometer (BD Immunocytometry Systems). At least 20,000 CD4$^+$ Vivid$^-$ events were gated for cell surface studies. Data were analyzed using FACSDiva software (BD Biosciences).

STAT-5/Bcl-2 staining

Various CD4$^+$CD25$^+$ cell populations (naive cells, activated cells, memory Tregs, and non-Tregs) were sorted at baseline based on CD4$^+$CD25$^+$ and CD45RA$^+$ expression. Cells were incubated or not with 10 ng/ml IL-7 for 20 min and then assessed for intracellular staining of phospho–STAT-5 FITC using flow cytometry (color LSR II flow cytometer; BD Immunocytometry Systems). Moreover, the same populations were incubated or not with IL-7 for a longer period (48 h) and analyzed for Bcl-2 expression.

Suppression assay

CD8$^+$ T cells were washed twice in PBS and stained for 8 min at 37°C with CFSE (final concentration, 0.5 μM) (Sigma-Aldrich, St. Louis, MO). CD8$^+$ CFSE-stained cells were then cultured in RPMI 1640 (Bio Whittaker Europe, Verviers, France) supplemented with 10% FBS (Life Technologies, Paisley, U.K.) and 1% penicillin streptomycin (Invitrogen) in a 96-well flat-bottom cell culture plate that had been coated for 2 h with 2 μg/ml immobilized anti-CD3 mAb (clone UCHT1; Beckman Coulter). Sorted Tregs (see above), incubated or not with IL-7, were added or not to the culture at a 1:4 Treg/CD8$^+$ T cell ratio. After incubation for 4–5 d, cells were washed, stained with CD8 PerCP, and analyzed by flow cytometry. The percentage of Treg-mediated inhibition was calculated as follows:

$$\frac{\%\text{ of cells without Tregs} - \%\text{ of cells with Tregs}}{\%\text{ of cells without Tregs}} \times 100$$

Cytokine measurement

Cytokine production in the supernatant of the different subpopulations of Tregs was evaluated using a Luminex assay (Bio-Plex 200; Bio-Rad, Berkley, CA). Purified CD4$^+$CD25$^+$ cells were incubated or not with IL-7 for 24 h; different Treg populations were sorted (see above) and incubated overnight with 2 μg/ml immobilized anti-CD3. Supernatant was collected and frozen at −80°C. The concentration of secreted IL-17 (Milliplex; Millipore, Billerica, MA) was measured in the supernatant (in pg/ml). The results were analyzed using Bio-Plex Manager 5 software (Bio-Rad).

Real-time PCR

Purified CD4$^+$CD25$^+$ cells were incubated or not with IL-7 for 24 h; different Treg populations were sorted (see above) and incubated for 24 h with 2 μg/ml immobilized anti-CD3. In some experiments, cells were incubated or not with 100 μM ATP (Sigma-Aldrich). Cells were harvested after 24 h, and total RNA was extracted using TRIzol reagent (Invitrogen). cDNA were synthesized using AffinityScript QPCR cDNA (StrataGen Systems). cDNA were then analyzed by RT-PCR using Brilliant II SYBR Green QPCR Master Mix (StrataGen; Agilent Technologies). The value of each sample was normalized to S14. The following primer sequences were used: S14: 5′-GGCAGACCGAGATGAATCCTCA-3′; 3′-CAGGTCCA-GGGGTCTTGGTCC-5′; IL-17: 5′-TGGAATCTCCACCGCAATGAGG-AC-3′ and 3′-AGCGTTGATGCAGCCCAAGTGG-5′; RORγt: 5′-GTCC-CGAGATGCTGTCAAGT-3′ and 3′-GACCACTGGTTCCTGTTGCT-5′; and P2X7: 5′-ACACCTTCCCTTTGCAGGGGAACT-3′ and 3′-TCGAT-GGGGCACCAGGCAGA-5′.

Statistical analysis

All experiments were repeated at least three times. The Student paired two-tailed *t* test was used to determine the significance between two groups of samples. A p value ≤ 0.05 was considered significant. All error bars represent SEM. All tests were performed using GraphPad Prism 4 software.

Results

IL-7 relieves the suppressive effect of memory Tregs

We first looked at the level of expression of CD127 on different Treg subpopulations (Fig. 1B) and analyzed their responsiveness to IL-7. Tregs were sorted from PBMCs according to their expression of CD45RA and CD25 (Fig. 1A). We isolated three populations of Tregs according to the description of Miyara et al. (1): nTregs (referred to hereafter as naive, population I: Foxp3^{++}, CD45RA$^+$, CD25^{++}), memory activated (referred to hereafter as activated, population II: Foxp3^{high}, CD45RA$^-$, CD25^{high}), and memory (referred to hereafter as memory, population III: Foxp3^{++}, CD45RA$^-$, CD25^{++}). We showed that all of these Treg subpopulations fully suppressed the proliferation of effector CD8 T cells (Fig. 1B). We isolated a fourth population (population IV; Foxp3^{low}, CD45RA$^-$, CD25$^+$) with no suppressive activity, which corresponded to activated T cells.

Although the expression of CD127 was lower in aTregs compared with other Treg populations (Fig. 2A) ($p < 0.05$, non-Tregs versus all other populations), all populations responded to IL-7 stimulation, which induces an increase in STAT-5 phosphorylation and Bcl-2

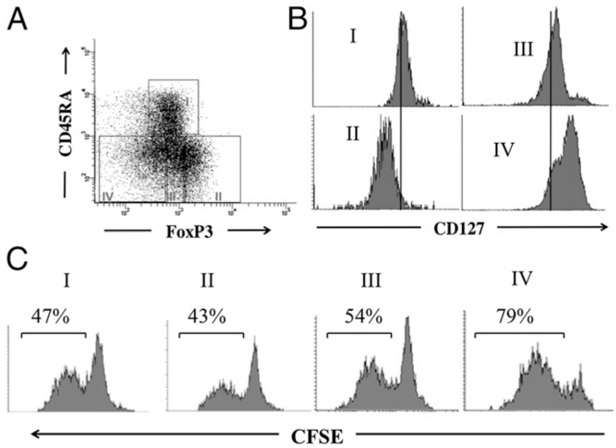

FIGURE 1. Delineation of CD4$^+$CD25$^+$ Treg populations. (**A**) Gating strategy of isolated CD4$^+$CD25$^+$ cells at baseline. Four subsets were defined based on the expression of CD45RA, FOXP3, and CD127 (population I = nTregs; population II = aTregs; population III = memory Tregs; population IV = non-Tregs). (**B**) Representative expression of CD127 on different gated populations of CD4$^+$CD25$^+$ cells [see (A)]. (**C**) Representative experiment showing the suppressive ability of sorted CD4$^+$CD25$^+$ cell populations [see (A)] in coculture with autologous CFSE-stained CD8$^+$ cells for 5 d in the presence of immobilized anti-CD3.

expression ($p < 0.05$, before versus after IL-7, for all populations) (Fig. 2B, 2C).

In contrast, IL-7 was unable to induce the proliferation of Treg populations (Fig. 1D). Next we checked whether IL-7 modulates the suppressive effect of Tregs. Treg subpopulations incubated for 24 h with IL-7 were cocultured with purified CD8 T cells at a 1:4 ratio. Although IL-7 did not influence the suppressive effect of nTregs (25% decrease after IL-7, p = NS), we observed a dramatic decrease in the suppressive effect of both aTregs (70% decrease, $p < 0.05$) and memory Tregs (99% decrease, $p < 0.05$) after IL-7 preincubation (Fig. 2E, Supplemental Fig. 1). Globally, these results show that CD127 is functional on human Treg subpopulations and that IL-7 relieves the suppressive effect of memory Tregs.

IL-7 decreases CD39 expression on memory Tregs

Next we looked at whether IL-7 was able to modify the phenotype of Treg subpopulations. We first examined the modulation of the expression of several well-characterized molecules (CTLA-4, GITR, CD39, and PDL1) involved in the Treg-mediated suppressive effect in Treg subpopulations after IL-7 incubation. IL-7 did not alter the expression of CTLA-4 on Tregs (Fig. 3A, Supplemental Fig. 2). We observed a slight, but insignificant, increase in PDL1 on memory Tregs ($p = 0.08$, before versus after IL-7) (Fig. 3B). GITR expression was increased on aTregs and memory Tregs after IL-7 exposure (aTregs, $p = 0.003$; memory Tregs, $p = 0.002$, before versus after IL-7) (Fig. 3C). Finally, we found that IL-7 induced a decrease in CD39 expression on activated and memory Tregs (activated $p < 0.001$; memory $p < 0.001$; between before and after IL-7) (Fig. 3D). Because IL-7 did not induce a significant proliferation of Tregs (Fig. 2D), we conclude that IL-7 modifies the expression of molecules involved in the suppressive functions of activated and memory Tregs rather than a proliferation of expressing or nonexpressing cells.

Memory, but not naive, Tregs incubated in the presence of IL-7 acquire a Th17 phenotype

We showed that IL-7 decreased CD39 expression on Treg subpopulations and concomitantly modified their suppressive effect. We next wanted to analyze whether decreased CD39 expression was

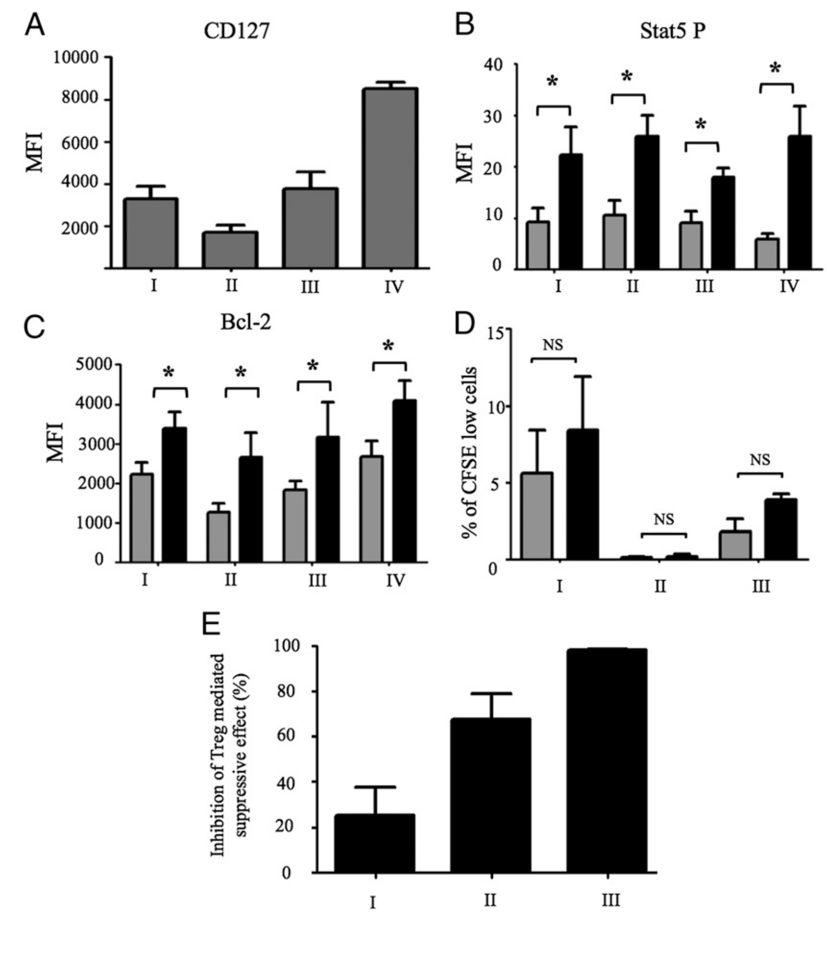

FIGURE 2. Effect of IL-7 on CD4$^+$CD25$^+$ cell populations. (**A**) Mean fluorescence intensity (MFI) expression of CD127 on the different CD4$^+$CD25$^+$ cell subsets (population I = nTregs; population II = aTregs; population III = memory Tregs; population IV = non-Tregs) (n = 5). (**B**) Sorted CD4$^+$CD25$^+$ cell subsets were incubated (black bars) or not (gray bars) with 10 ng/ml of IL-7. Expression of phosphorylated STAT-5 was assessed by flow cytometry analysis after 20 min (n = 3). (**C**) CD4$^+$CD25$^+$ cells were sorted and cultured as in (B). Expression of Bcl-2 was assessed by flow cytometry analysis after 48 h (n = 3). (**D**) CD4$^+$CD25$^+$ cells were sorted and stained with CFSE on day 0. CFSE staining was assessed after 5 d of culture with (black bars) or without (gray bars) IL-7 (n = 3). (**E**) Suppression assay showing percentage inhibition of effector T cell proliferation by different Treg populations after IL-7 treatment and then cocultured with CFSE-labeled effector CD8$^+$ T cells. Flow cytometric analysis was performed at day 5. The percentage of cells that was CFSE low was gated on CD8$^+$ T cell population (n = 4). Data are mean ± SEM. *$p < 0.05$, paired t test.

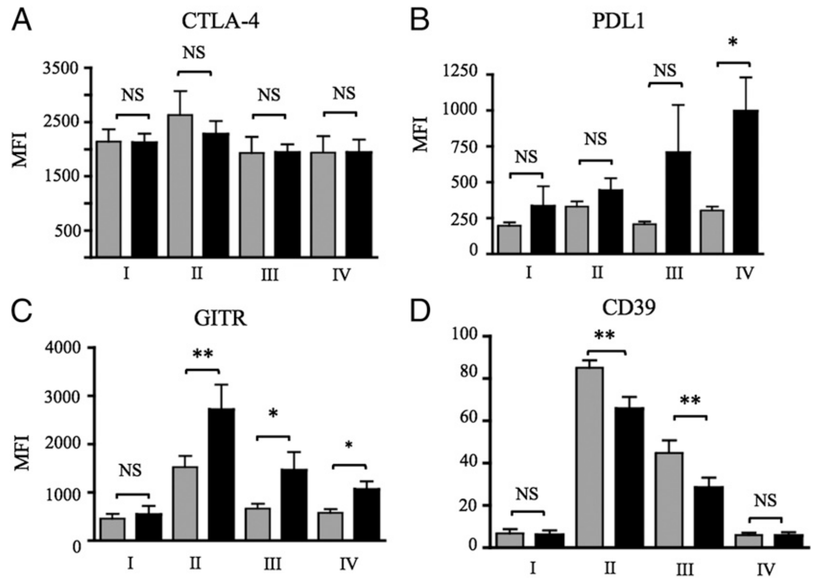

FIGURE 3. IL-7 decreases the expression of CD39 on memory Treg subsets. CTLA-4 (**A**), PDL1 (**B**), GITR (**C**), and CD39 (**D**) expression on the different CD4$^+$ CD25$^+$ cell subsets (see Fig. 1) assessed by flow cytometry analysis after a 24-h culture period with (black bars) or without (gray bars) IL-7 (10 ng/ml). Data are mean ± SEM (n = 9). *$p < 0.05$, **$p < 0.01$, paired t test.

the only impact of IL-7 signaling on Tregs and whether this cytokine could change the fate of these cells, redirecting them toward a Th17 phenotype, as recently described for other cytokines (23). We first analyzed the production of IL-17 by Luminex assay (*Materials and Methods*). We found that IL-7 dramatically increased the production of IL-17 by activated and memory Tregs, whereas no change was noted for nTregs (Fig. 4A). We then analyzed the expression of IL-17 and RORγt mRNA, a key transcription factor for IL-17 expression, by Tregs after IL-7 exposure, focusing on the naive and activated populations. IL-7 induced an increase in both IL-17 and RORγt mRNA in aTregs, whereas it still had no effect on nTregs (Fig. 4B, 4C). Altogether, these results indicated that IL-7 is able to modify the phenotype of aTregs, decreasing their CD39 expression and redirecting them toward a Th17 phenotype through an increased expression of RORγt.

IL-7 modulates memory Treg function through dual modification of the CD39/ATP axis

We next investigated the functional consequences of the effects of IL-7 on the expression of CD39 on activated and memory Tregs and whether the ATP axis was involved in the shift of Tregs toward Th17 cells in the presence of IL-7. For this experiment, aTregs were cultured in the presence of IL-7, with (or without) anti-CD39 Abs blocking CD39 enzymatic function and ATP. We looked at the expression of IL-17 and RORγt mRNAs under these culture conditions. Anti-CD39 Ab, alone or in combination with ATP, was not able to modify RORγt expression, and it had a slight effect on IL-17 expression (Fig. 5A, 5B). In contrast, the adjunction of IL-7 to ATP induced a dramatic increase in the expression of both IL-17 and RORγt mRNA (Fig. 5C, 5D). Next, we looked at the expression of P2X7, a key receptor of ATP (23), and found a dramatic increase in its expression on aTregs in the presence of IL-7 (Fig. 5E). We used the P2X7 antagonist, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPAD), to confirm that this effect could explain the increased production of IL-17 by aTregs treated with IL-7 (27). PPAD completely blocked the expression of the mRNA IL-17 and RORγt induced by IL-7 on aTregs (Fig. 6A, 6B). In contrast, PPAD was unable to reverse the suppressive effect of aTregs after IL-7 incubation (Fig. 5C). Altogether, these results indicated that IL-7 has a dual role on aTregs: it decreases

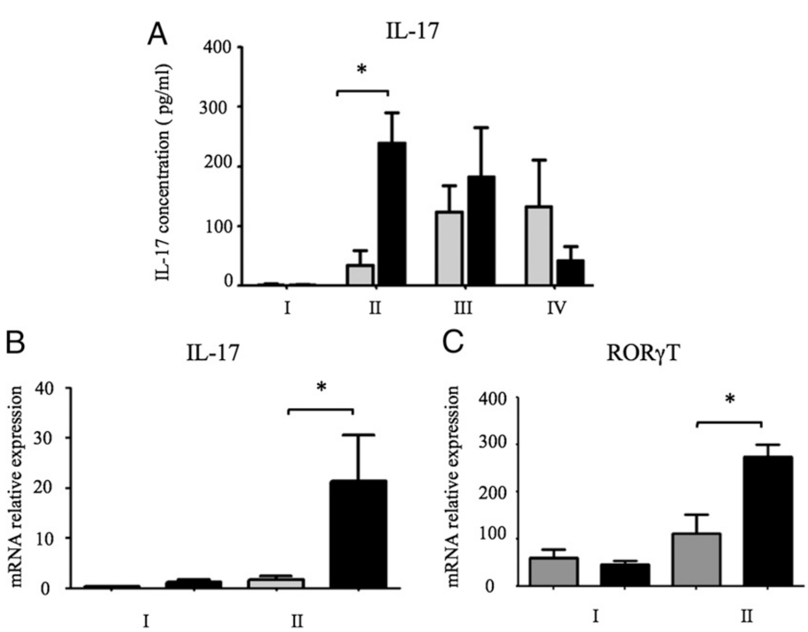

FIGURE 4. IL-7 redirects memory Tregs toward a Th17 phenotype. (**A**) Sorted CD4$^+$CD25$^+$ cell subsets (see Fig. 1) were incubated (black bars) or not (gray bars) with IL-7 for 24 h and then activated overnight with 2 μg/ml anti-CD3. Supernatants were analyzed for IL-17 production using Luminex technology. (**B**) RNA from the same experiments was extracted, and real-time PCR was performed to analyze IL-17 expression in nTregs (I) and aTregs (II). (**C**) Same as in (B) for RORγt expression. Data are mean ± SEM (n = 4). *$p < 0.05$, paired t test.

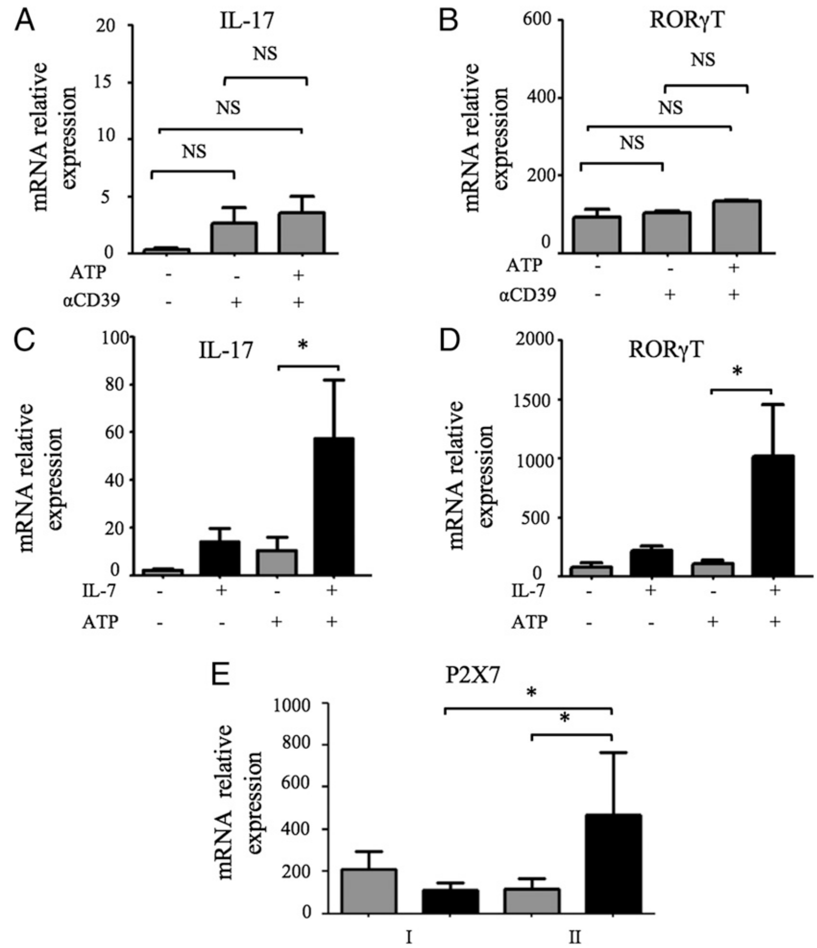

FIGURE 5. ATP potentiates the effects of IL-7 on memory Tregs. (**A**) aTregs were sorted and incubated or not with purified 2 μg/ml anti-CD39 mAb (A1, Ozyme) at 37°C and/or with or without ATP 100 μM for 24 h. Total RNA was extracted, and real-time PCR was performed to analyze IL-17 expression. (**B**) Same as in (A) for RORγt expression. (**C**) Sorted memory Tregs were incubated (black bars) or not (gray bars) with IL-7 (10 ng/ml) for 24 h and cultured for another 24 h with or without ATP (100 μM). Total RNA was extracted, and real-time PCR was performed to analyze IL-17 expression. (**D**) Same as in (C) for RORγt expression. (**E**) nTregs (I) and aTregs (II) were incubated (black bars) or not (gray bars) with IL-7 for 24 h. P2X7 expression was assessed by RT-PCR. Data are mean ± SEM (n = 4). *$p < 0.05$, paired t test.

their suppressive effect through a decrease in CD39 expression and redirects them toward a Th17 phenotype through an increase in the expression of the P2X7 receptor.

Treatment with IL-7 decreases the percentage of memory Tregs and their expression of CD39 in vivo during HIV infection

We recently showed the potential impact of IL-7 therapy during HIV infection (26, 28). We first studied the in vitro effect of IL-7 on Treg subsets from HIV-infected patients with undetectable viral load under c-ART. We found that IL-7 induced a decrease in CD39 expression on aTregs, as previously shown for HIV^- subjects. A decrease was also observed in memory Tregs, but this difference was not significant (Supplemental Fig. 3).

Then we analyzed the effect of IL-7 treatment on the evolution of Treg subsets in vivo. Cells were obtained from nine patients included in the INSPIRE trial (26). Treatment with IL-7 did not

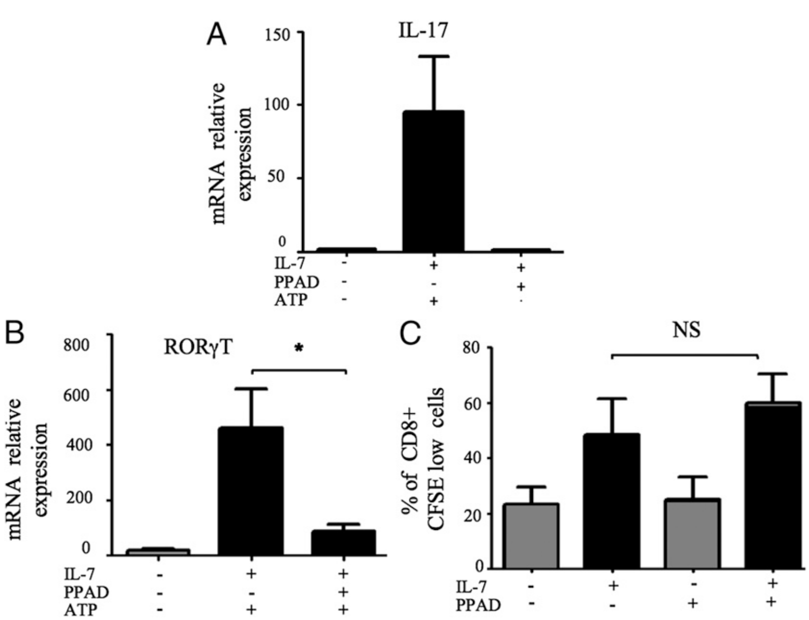

FIGURE 6. IL-7 increases the expression of P2X7 receptor on aTregs and redirects them toward a Th17 phenotype. (**A**) Activated sorted Tregs were incubated (black bars) or not (gray bars) with IL-7 for 24 h. Cells were also preincubated or not with ATP receptor blocker PPAD (20 μM; Sigma-Aldrich) for 2 h at 37°C and/or ATP (100 μM). IL-17 expression was assessed by RT-PCR analysis. (**B**) Same as (A) for RORγt expression. (**C**) Activated Tregs were sorted and incubated or not with 10 ng/ml IL-7 for 24 h and with ATP receptor blocker PPAD (20 μM) for 2 h and cocultured with autologous CFSE-stained $CD8^+$ cells. The percentage of $CD8^+CFSE^{low}$ cells was analyzed at 5 d. Data are mean ± SEM (n = 3). *$p < 0.05$, paired t test.

change the percentage of the total Treg population, whereas it increased the percentage of memory and activated cells among Tregs (data not shown). Tregs were purified from blood samples obtained before and after 12 wk of treatment with IL-7. First, we confirmed that in vitro incubation with IL-7 was able to decrease CD39 expression in memory Tregs (Fig. 7A). In eight patients for whom cells were available at day 0 and week 12, IL-7 therapy led to a decrease in CD39 expression on memory Tregs (Fig. 7B). Moreover, we found a correlation between the in vitro and ex vivo effects of IL-7 on CD39 expression on Tregs in the four patients for whom we had sufficient material to perform ex vivo and in vitro studies (these patients appear as ■, ●, △, and × in Fig. 7A, 7B). Finally, we were able to perform mRNA analyses from purified Tregs from five patients in the INSPIRE study. Compared with baseline, we found a significant increase in both RORγt (Fig. 7C, $p = 0.05$) and P2X7 (Fig. 7D, $p < 0.01$) in Treg populations following IL-7 therapy.

Discussion

$FOXP3^+$ Tregs are a heterogeneous population of $CD4^+$ T cells that includes nTregs and aTregs, which were recently classified into diverse subpopulations by Miyara et al. (1). These populations seem to respond to different stimuli for their induction and survival (28). Although IL-2 was thought to be specifically required for the generation and maintenance of nTregs, experiments in IL-$2^{-/-}$ and $CD25^{-/-}$ discussed this fact (29). Gene knockout of IL-2Rγc leads to a complete defect in $CD4^+Foxp3^+$ Tregs (30), a phenomenon in which the transcription factor STAT-5 is critically involved (31). Several recent reports indicated that IL-7 could be a determinant for the survival of Tregs (9–19). Like IL-2 and IL-15, IL-7 induces STAT-5 phosphorylation of Tregs in vitro (12, 14, 32). In mice, in vivo experiments showed that IL-7 primarily affects nTreg development (14, 16, 33). However, discrepancies about the role of IL-7 in Treg homeostasis remain. Some reports did not find a role for IL-17 in aTreg homeostasis (14), whereas others showed that it increases Treg numbers by inducing thymic-independent Treg peripheral expansion (17). Recently, Heninger et al. (34) showed that IL-7 could modify Treg functions. IL-7 induced phosphorylation of STAT-5 in naive and memory Tregs; promoted the proliferation of naive, but not memory, populations; and blocked the ability of Tregs to mediate suppression of effector T cells either after nonspecific or specific activation. However, the mechanisms involved in these phenomena remain unknown.

Our results significantly extend these previous studies. First, we report that IL-7, despite signaling the CD127 receptor on all Treg populations, did not increase the proliferation of these cells. This discrepancy with regard to other studies could result from the use of different coactivation systems in our study compared with the more potent activation signals delivered by anti-CD3 and anti-CD28 Abs. Second, we show that IL-7 decreases the suppressive effect of memory Tregs (up to 90%) but not nTregs (<25%). We clearly show that this effect relies on the modulation of the expression of the ectoenzyme CD39. As previously shown by our group (3) and other investigators (35), Tregs in mice constitutively express CD39, whereas the proportion of human $CD39^+$ Tregs appears to be highly variable. We found that, although IL-7 signals both naive and memory Tregs by increasing STAT-5 phosphorylation and Bcl-2 expression, this cytokine did not relieve the suppressive effect of nTregs that relies on their poor CD39 expression. CD39 and CD73 enzymes present on $CD4^+$ $CD25^{high}$ T cells generate adenosine, a potent suppressor of effector T cell proliferation and function. $CD39^+$ Tregs are thought to be involved in many clinical situations in humans, such as HIV and hepatitis B virus infections (3, 36), renal grafts, and cancer. CD39 blockade in mice (37) or in humans (3) was shown to impair their suppressive function. Our data contribute to increase the arsenal of tools that may affect Treg function by modulation of CD39 expression through external signals.

Recent data showed that the differentiation program of $FoxP3^+$ Tregs is not fixed. Depending on the presence of external factors, these cells are able to differentiate into Tfh or Th17 cells (25). The presence of IL-6, an inflammatory cytokine produced by APCs, blocked the Treg-mediated suppressive effect (38), probably because of their conversion to Th17 cells (23). In addition to cytokines, other factors are known to be critical for peripheral Th17 differentiation. In mice, ATP produced by commensal bacteria promoted the presence of Th17 in the intestine and exacerbated T cell–mediated colitis (39). Recently Schenk et al. (23) showed that ATP mediates Treg conversion to Th17 cells through signaling via the P2X7 receptor, which can be increased by IL-6. We found that another cytokine, IL-7, redirects aTregs toward a Th17

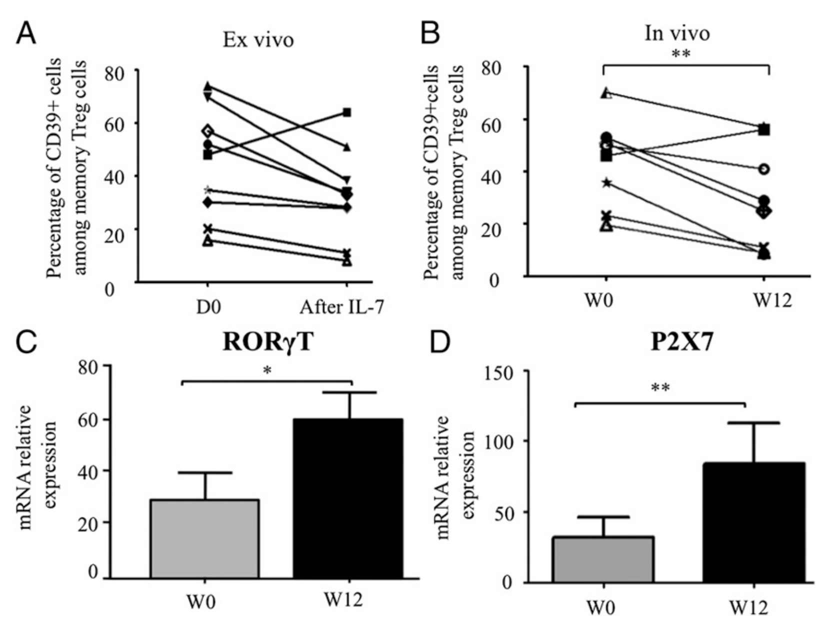

FIGURE 7. Effects of IL-7 therapy on Treg memory subsets. (**A**) $CD4^+CD25^{high}$ Tregs were isolated from PBMCs of nine HIV^+ patients included in the INSPIRE study. Tregs were incubated in vitro with 10 ng/ml of IL-7 for 24 h. The percentage of $CD39^+$ cells among memory Tregs was assessed by flow cytometry before and after IL-7 incubation. (**B**) The percentage of $CD39^+$ cells among memory $CD4^+CD25^{high}$ Tregs was assessed in eight HIV^+ patients, who were included in the INSPIRE study, before and after IL-7 treatment. $CD4^+CD25^{high}$ Tregs were purified in five HIV^+ patients included in the INSPIRE study. Total RNA was extracted, and real-time PCR was performed to analyze RORγt (**C**) and P2X7 (**D**) mRNA expression before (gray bars) and 12 wk after (black bars) IL-7 treatment. Data are mean ± SEM. $*p < 0.05$, $**p < 0.01$, paired t test.

phenotype through an increase in P2X7 expression. In contrast to the study by Schenk et al. (23), we found that ATP/P2X7 seems to have minimal involvement in Treg-mediated suppressive activity (Fig. 5D). Therefore, we propose that IL-7 could have dual, but synergistic, effects through downmodulation of CD39 and an increase in P2X7 receptor. Both lead to an increase in the ATP-mediated effect, tipping the balance toward Th17 conversion.

What is the physiological relevance of this effect of IL-7 on Treg function? Thymus, skin, and the intestine are major sources of IL-7 (40). During the steady state, the majority of Th17 cells are located in the intestine where they are educated and can expand. Commensal bacteria may influence Th17 differentiation locally through ATP release (39), but they also may induce Tregs through TCR recognition of commensal Ags (41). We speculate that in physiology, IL-7 produced by intestinal epithelial cells could, in association with commensal bacteria of the microbiota, regulate the balance between aTregs and Th17 cells (42).

Finally, we (26, 43, 44) and other investigators (45–48) showed in several clinical trials in cancer or HIV that IL-7 increases Treg numbers, whereas their percentage among T cells decreases. Pelligrini et al. (45) demonstrated that IL-7 administration during chronic viral infection leads to viral control. Notably, they found a large shift in the cytokine profile, an increase in the levels of IL-6 and IL-17, and a decrease in the level of immunosuppressive TGF-β. We showed in this study that IL-7 therapy decreases CD39 membrane expression and promotes expression of RORγt and P2X7 in aTregs, which sum up what we observed in vitro. Although these effects were not observed in all patients, overall we found an association between IL-7–mediated CD39 downregulation in vivo and in vitro. These differences could be the consequence of the presence of different polymorphisms of the IL-7R, which may induce differences in the responsiveness to IL-7 (44).

In conclusion, the results from this study extend our knowledge about the role of IL-7 in T cell homeostasis by dissecting its effects on subpopulations of memory Tregs. In contrast, these results contribute to improved characterization of these Treg subpopulations, showing that, beyond their phenotypic and functional heterogeneity, human Tregs could respond differently to external signals. Finally, convergent data show the modulation of memory Treg function through the ATP/CD39 axis. Previous in vitro studies that attempted to modulate these functions using blocking Abs or chemical products were performed by our group (3) and other investigators (35). We provide evidence that a therapeutic approach using IL-7, a cytokine in phase II clinical development, may also impact Treg phenotype comparably to in vitro effects. These observations reinforce the potential use of IL-7 in HIV infection characterized by an altered Treg/Th17 cell ratio.

Disclosures

S.B. and T.C. are employees of Cytheris, a company that develops IL-7 for therapeutic use. The other authors have no financial conflicts of interest.

References

1. Miyara, M., Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, et al. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30: 899–911.
2. Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S. I. Alexander, R. Nanan, et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 203: 1693–1700.
3. Nikolova, M., M. Carriere, M. A. Jenabian, S. Limou, M. Younas, A. Kök, S. Huë, N. Seddiki, A. Hulin, O. Delaneau, et al. 2011. CD39/adenosine pathway is involved in AIDS progression. *PLoS Pathog.* 7: e1002110.
4. Borsellino, G., M. Kleinewietfeld, D. Di Mitri, A. Sternjak, A. Diamantini, R. Giometto, S. Höpner, D. Centonze, G. Bernardi, M. L. Dell'Acqua, et al. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110: 1225–1232.
5. Mandapathil, M., S. Lang, E. Gorelik, and T. L. Whiteside. 2009. Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression. *J. Immunol. Methods* 346: 55–63.
6. Abrams, D., Y. Lévy, M. H. Losso, A. Babiker, G. Collins, D. A. Cooper, J. Darbyshire, S. Emery, L. Fox, F. Gordin, et al; INSIGHT-ESPRIT Study Group; SILCAAT Scientific Committee. 2009. Interleukin-2 therapy in patients with HIV infection. *N. Engl. J. Med.* 361: 1548–1559.
7. Weiss, L., F. A. Letimier, M. Carriere, S. Maiella, V. Donkova-Petrini, B. Targat, A. Benecke, L. Rogge, and Y. Levy. 2010. In vivo expansion of naive and activated CD4+CD25+FOXP3+ regulatory T cell populations in interleukin-2-treated HIV patients. *Proc. Natl. Acad. Sci. USA* 107: 10632–10637.
8. Saadoun, D., M. Rosenzwajg, F. Joly, A. Six, F. Carrat, V. Thibault, D. Sene, P. Cacoub, and D. Klatzmann. 2011. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N. Engl. J. Med.* 365: 2067–2077.
9. Bayer, A. L., J. Y. Lee, A. de la Barrera, C. D. Surh, and T. R. Malek. 2008. A function for IL-7R for CD4+CD25+Foxp3+ T regulatory cells. *J. Immunol.* 181: 225–234.
10. Chen, X., L. Fang, S. Song, T. B. Guo, A. Liu, and J. Z. Zhang. 2009. Thymic regulation of autoimmune disease by accelerated differentiation of Foxp3+ regulatory T cells through IL-7 signaling pathway. *J. Immunol.* 183: 6135–6144.
11. Haas, J., M. Korporal, A. Schwarz, B. Balint, and B. Wildemann. 2011. The interleukin-7 receptor α chain contributes to altered homeostasis of regulatory T cells in multiple sclerosis. *Eur. J. Immunol.* 41: 845–853.
12. Harnaha, J., J. Machen, M. Wright, R. Lakomy, A. Styche, M. Trucco, S. Makaroun, and N. Giannoukakis. 2006. Interleukin-7 is a survival factor for CD4+ CD25+ T-cells and is expressed by diabetes-suppressive dendritic cells. *Diabetes* 55: 158–170.
13. Liu, X., S. Leung, C. Wang, Z. Tan, J. Wang, T. B. Guo, L. Fang, Y. Zhao, B. Wan, X. Qin, et al. 2010. Crucial role of interleukin-7 in T helper type 17 survival and expansion in autoimmune disease. *Nat. Med.* 16: 191–197.
14. Mazzucchelli, R., J. A. Hixon, R. Spolski, X. Chen, W. Q. Li, V. L. Hall, J. Willette-Brown, A. A. Hurwitz, W. J. Leonard, and S. K. Durum. 2008. Development of regulatory T cells requires IL-7Ralpha stimulation by IL-7 or TSLP. *Blood* 112: 3283–3292.
15. Pandiyan, P., and M. J. Lenardo. 2008. The control of CD4+CD25+Foxp3+ regulatory T cell survival. *Biol. Direct* 3: 6.
16. Peffault de Latour, R., H. C. Dujardin, F. Mishellany, O. Burlen-Defranoux, J. Zuber, R. Marques, J. Di Santo, A. Cumano, P. Vieira, and A. Bandeira. 2006. Ontogeny, function, and peripheral homeostasis of regulatory T cells in the absence of interleukin-7. *Blood* 108: 2300–2306.
17. Simonetta, F., N. Gestermann, K. Z. Martinet, M. Boniotto, P. Tissières, B. Seddon, and C. Bourgeois. 2012. Interleukin-7 influences FOXP3+CD4+ regulatory T cells peripheral homeostasis. *PLoS ONE* 7: e36596.
18. Vang, K. B., J. Yang, S. A. Mahmud, M. A. Burchill, A. L. Vegoe, and M. A. Farrar. 2008. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development. *J. Immunol.* 181: 3285–3290.
19. Wuest, T. Y., J. Willette-Brown, S. K. Durum, and A. A. Hurwitz. 2008. The influence of IL-2 family cytokines on activation and function of naturally occurring regulatory T cells. *J. Leukoc. Biol.* 84: 973–980.
20. da Silva Martins, M., and C. A. Piccirillo. 2012. Functional stability of Foxp3+ regulatory T cells. *Trends Mol. Med.* 18: 454–462.
21. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.
22. Duarte, J. H., S. Zelenay, M. L. Bergman, A. C. Martins, and J. Demengeot. 2009. Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions. *Eur. J. Immunol.* 39: 948–955.
23. Schenk, U., M. Frascoli, M. Proietti, R. Geffers, E. Traggiai, J. Buer, C. Ricordi, A. M. Westendorf, and F. Grassi. 2011. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. *Sci. Signal.* 4: ra12.
24. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, et al. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29: 44–56.
25. Zhou, L., M. M. Chong, and D. R. Littman. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 30: 646–655.
26. Lévy, Y., I. Sereti, G. Tambussi, J. P. Routy, J. D. Lelièvre, J. F. Delfraissy, J. M. Molina, M. Fischl, C. Goujard, B. Rodriguez, et al. 2012. Effects of recombinant human interleukin 7 on T-cell recovery and thymic output in HIV-infected patients receiving antiretroviral therapy: results of a phase I/IIa randomized, placebo-controlled, multicenter study. *Clin. Infect. Dis.* 55: 291–300.
27. North, R. A., and A. Surprenant. 2000. Pharmacology of cloned P2X receptors. *Annu. Rev. Pharmacol. Toxicol.* 40: 563–580.
28. Wan, Y. Y., and R. A. Flavell. 2006. The roles for cytokines in the generation and maintenance of regulatory T cells. *Immunol. Rev.* 212: 114–130.
29. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6: 1142–1151.
30. Malek, T. R., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17: 167–178.

31. Burchill, M. A., J. Yang, C. Vogtenhuber, B. R. Blazar, and M. A. Farrar. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J. Immunol.* 178: 280–290.
32. Juffroy, O., F. Bugault, O. Lambotte, I. Landires, J. P. Viard, L. Niel, A. Fontanet, J. F. Delfraissy, J. Thèze, and L. A. Chakrabarti. 2010. Dual mechanism of impairment of interleukin-7 (IL-7) responses in human immunodeficiency virus infection: decreased IL-7 binding and abnormal activation of the JAK/STAT5 pathway. *J. Virol.* 84: 96–108.
33. Bayer, A. L., A. Yu, D. Adeegbe, and T. R. Malek. 2005. Essential role for interleukin-2 for CD4(+)CD25(+) T regulatory cell development during the neonatal period. *J. Exp. Med.* 201: 769–777.
34. Heninger, A. K., A. Theil, C. Wilhelm, C. Petzold, N. Huebel, K. Kretschmer, E. Bonifacio, and P. Monti. 2012. IL-7 abrogates suppressive activity of human CD4+CD25+FOXP3+ regulatory T cells and allows expansion of alloreactive and autoreactive T cells. *J. Immunol.* 189: 5649–5658.
35. Schuler, P. J., M. Harasymczuk, B. Schilling, S. Lang, and T. L. Whiteside. 2011. Separation of human CD4+CD39+ T cells by magnetic beads reveals two phenotypically and functionally different subsets. *J. Immunol. Methods* 369: 59–68.
36. Tang, Y., L. Jiang, Y. Zheng, B. Ni, and Y. Wu. 2012. Expression of CD39 on FoxP3+ T regulatory cells correlates with progression of HBV infection. *BMC Immunol.* 13: 17.
37. Deaglio, S., K. M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J. F. Chen, K. Enjyoji, J. Linden, M. Oukka, et al. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* 204: 1257–1265.
38. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299: 1033–1036.
39. Atarashi, K., J. Nishimura, T. Shima, Y. Umesaki, M. Yamamoto, M. Onoue, H. Yagita, N. Ishii, R. Evans, K. Honda, and K. Takeda. 2008. ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455: 808–812.
40. Shalapour, S., K. Deiser, A. A. Kühl, R. Glauben, S. M. Krug, A. Fischer, O. Sercan, S. Chappaz, S. Bereswill, M. M. Heimesaat, et al. 2012. Interleukin-7 links T lymphocyte and intestinal epithelial cell homeostasis. *PLoS ONE* 7: e31939.
41. Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Müller, T. Sparwasser, R. Förster, and O. Pabst. 2011. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* 34: 237–246.
42. Huber, S., N. Gagliani, and R. A. Flavell. 2012. Life, death, and miracles: Th17 cells in the intestine. *Eur. J. Immunol.* 42: 2238–2245.
43. Levy, Y., C. Lacabaratz, L. Weiss, J. P. Viard, C. Goujard, J. D. Lelièvre, F. Boué, J. M. Molina, C. Rouzioux, V. Avettand-Fénoêl, et al. 2009. Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. *J. Clin. Invest.* 119: 997–1007.
44. Limou, S., G. Melica, C. Coulonges, J. D. Lelièvre, H. Do, S. McGinn, I. G. Gut, Y. Lévy, and J. F. Zagury. 2012. Identification of IL7RA risk alleles for rapid progression during HIV-1 infection: a comprehensive study in the GRIV cohort. *Curr. HIV Res.* 10: 143–150.
45. Pellegrini, M., T. Calzascia, J. G. Toe, S. P. Preston, A. E. Lin, A. R. Elford, A. Shahinian, P. A. Lang, K. S. Lang, M. Morre, et al. 2011. IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology. *Cell* 144: 601–613.
46. Rosenberg, S. A., C. Sportès, M. Ahmadzadeh, T. J. Fry, L. T. Ngo, S. L. Schwarz, M. Stetler-Stevenson, K. E. Morton, S. A. Mavroukakis, M. Morre, et al. 2006. IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. *J. Immunother.* 29: 313–319.
47. Sereti, I., R. M. Dunham, J. Spritzler, E. Aga, M. A. Proschan, K. Medvik, C. A. Battaglia, A. L. Landay, S. Pahwa, M. A. Fischl, et al; ACTG 5214 Study Team. 2009. IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. *Blood* 113: 6304–6314.
48. Sportès, C., F. T. Hakim, S. A. Memon, H. Zhang, K. S. Chua, M. R. Brown, T. A. Fleisher, M. C. Krumlauf, R. R. Babb, C. K. Chow, et al. 2008. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naive T cell subsets. *J. Exp. Med.* 205: 1701–1714.