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A Negative Feedback Loop Mediated by STAT3 Limits Human Th17 Responses

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The transcription factor STAT3 is critically required for the differentiation of Th17 cells, a T cell subset involved in various chronic inflammatory diseases. In this article, we report that STAT3 also drives a negative-feedback loop that limits the formation of IL-17-producing T cells within a memory population. By activating human memory CD4+CD45RO+ T cells at a high density (HiD) or a low density (LoD) in the presence of the pro-Th17 cytokines IL-1β, IL-23, and TGF-β, we observed that the numbers of Th17 cells were significantly higher under LoD conditions. Assessment of STAT3 phosphorylation revealed a more rapid and stronger STAT3 activation in HiD cells than in LoD cells. Transient inhibition of active STAT3 in HiD cultures significantly enhanced Th17 cell numbers. Expression of the STAT3-regulated ectonucleotidase CD39, which catalyzes ATP hydrolysis, was higher in HiD, than in LoD, cell cultures. Interestingly, inhibition of CD39 ectonucleotidase activity enhanced Th17 responses under HiD conditions. Conversely, blocking the ATP receptor P2X7 reduced Th17 responses in LoD cultures. These data suggest that STAT3 negatively regulates Th17 cells by limiting the availability of ATP. This negative-feedback loop may provide a safety mechanism to limit tissue damage by Th17 cells during chronic inflammation. Furthermore, our results have relevance for the design of novel immunotherapeutics that target the STAT3-signaling pathway, because inhibition of this pathway may enhance, rather than suppress, memory Th17 responses. The Journal of Immunology, 2014, 193: 000–000.
used on memory CD4+ T cells because induction or augmentation of IL-17 production in these cells has been proposed to contribute to the pathogenesis of chronic inflammatory disorders (28–30). Unexpectedly, we found a negative-feedback loop operating through STAT3 under high T cell density conditions. This negative-feedback loop may serve as a protective mechanism to restrain Th17 cells during overly exuberant inflammatory responses.

Materials and Methods

Isolation of memory CD4+ T cells

Human samples were obtained with informed consent in accordance with the Declaration of Helsinki and after approval by the Newcastle and North Tyneside Research Ethics Committee 2. PBMCs were isolated by density centrifugation on Lymphoprep (Axis-Shield Diagnostics) from Leukocyte Reduction System cones from platelet donations. CD4+CD45RO+ memory T cells were isolated by negative selection using an EasySep Human Memory CD4+ T Cell Enrichment Kit (STEMCELL Technologies). Enrichment of CD4+CD45RO+ cells was routinely >95%, as determined by flow cytometry (data not shown).

Activation of T cells

Memory CD4+ T cells were cultured in IMDM (Sigma-Aldrich) containing 10% (v/v) serum replacement (Invitrogen) and supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. A total of 1 × 10^6 high-density (HiD) or 0.0625 × 10^6 low-density (LoD) T cells/ml was activated with CD3/CD28 T cell expander Dynabeads (Invitrogen) at a 1:1 bead:T cell ratio in the presence of the following pro-Th17 cytokine mixture: IL-1β (10 ng/ml; PeproTech), IL-23 (10 ng/ml; R&D Systems), and TGF-β (10 ng/ml; PeproTech). Alternatively, T cells were activated by autologous monocyte-derived dendritic cells (moDCs; 1:10 T cell:moDC ratio) that had been activated with peptides and Candida albicans for 24 h prior to coculture with T cells. T cells were cultured for 6 d at 37°C, in 5% CO2, and IL-2 was refreshed on day 3 with the addition of 10 U/ml IL-2 (Proliekin; Novartis). Intracellular cytokine staining was performed as described below. For selected cultures, the STAT3 inhibitor S31-201 (50 µM; Tocris) was added on days 0 and 3, and ruxolitinib (50 µM; Calbiochem) was added to HiD cultures on days 0 and 1, the JAK1/2 inhibitor 10:1 T cell/moDC and TGF-β, respectively, were measured.

Intracellular cytokine staining

Human T cells were activated with PMA (10 ng/ml) and ionomycin (1 µg/ml; both from Sigma-Aldrich), 1 h later, brefeldin A (10 µg/ml) was added for 4 h. Cells were harvested and stained with Via-Probe (BD Bioscience) before being fixed and permeabilized using appropriate buffers (Foxp3 staining buffer set; eBioscience). To reduce background staining, the cells were blocked with 200 µg/ml mouse serum (IgG; Sigma-Aldrich) for 15 min before the addition of Abs. The following Abs were used: IFN-γ–FITC (4S.B3) and IL-17–Alexa Fluor 647 (eBioscience). Cells were incubated at 4°C for 30 min, washed, and resuspended in FACS buffer. Data were collected using a FACSCanto II (Becton Dickinson) and analyzed using FlowJo Version 8.7.1 (TreeStar). Dead cells were included from the total acquisition population using Via-Probe, and doublet exclusion was performed on the basis of the side scatter height versus side scatter area. Live singlet cells were assessed for intracellular cytokine expression using fluorescence-minus-one to set negative gates.

Real-time PCR

Total RNA was extracted from T cells using TRIzol reagent, and cDNA was reverse transcribed using Superscript III Reverse Transcriptase (both from Invitrogen). RORc forward 5'-CAGCGCTCCAACATCTTCT-3' and reverse 5'-CCACATCTCCACATCGACT-3' primers were purchased from Sigma-Aldrich. Gene expression was measured by SYBR Green (Invitrogen) quantitative real-time PCR. Relative RORc gene expression was normalized to RORα gene expression at day 0.

ELISA

Day-6 T cells from HiD and LoD cultures were washed and resuspended at 1 × 10^6 cells/ml in IMDM with 10% v/v serum replacement, rested for 1 h, and restimulated with PMA (10 ng/ml) and ionomycin (1 µg/ml) for 24 h. IL-17 and IFN-γ cytokine concentrations were determined in supernatants by specific sandwich ELISA (IL-17A: eBioscience; IFN-γ: BD Pharmingen). In other experiments, supernatants of HiD and LoD cultures were harvested at 48 h, and levels of IL-6, IL-10, TNF-α, and IFN-γ were determined by specific sandwich ELISA (all from BD Pharmingen).

STAT3 Phosflow

Cells were harvested, fixed, and permeabilized using appropriate buffers (Cytofix Fixation Buffer and Phosflow Perm Buffer III; BD Bioscience) at the time points indicated. To reduce background staining, the cells were blocked with 200 µg/ml mouse serum (Sigma-Aldrich) for 15 min prior to the addition of Abs. The following Abs were used: phospho-STAT3 (clone Tyr705-20; Alexa Fluor 647; 4/P-STAT3) and phospho-STAT1 Tyr701-20; Alexa Fluor 647 (4a; both from BD). Cells were incubated at 37°C for 40 min, washed, and resuspended in Ca²⁺/Mg²⁺-containing FACS buffer. Data were collected, gated, and analyzed as described for "intracellular cytokine staining" above.

Western blotting

T cells were cultured under HiD and LoD conditions in the presence or absence of pro-Th17 cytokines for 24 h. Whole-cell extracts were prepared with Cell Lytic. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked in 5% dried milk and 0.05% Tween in Tris-buffered salt solution. Membranes were probed with anti-STAT3 (clone) and anti-phospho STAT3 (clone Tyr705-20; mAb (clone D3A7), followed by HRP-conjugated anti-rabbit Ab (all from Cell Signaling). Proteins were visualized using the Immobilon Western detection system (Millipore).

Statistical analysis

Statistics were performed using Prism 5.0 software (GraphPad).

Results

Activation of T cells at LoD promotes Th17, but not Th1, responses

To assess the effect of cell density on the generation of Th17 responses, human CD4+CD45RO+ memory T cells were activated with CD3/CD28 expander beads at a 1:1 bead/T cell ratio and cultured at HiD (1 × 10^6 cells/ml) or LoD (6.25 × 10^5 cells/ml) in the presence of pro-Th17 cytokines. After 6 d of culture, T cells were restimulated with PMA/ionomycin, and the Th1 and Th17 signature cytokines, IL-17 and IFN-γ, respectively, were measured. LoD stimulation of T cells led to a marked and significant increase in the percentage of IL-17–producing T cells, whereas the proportion of IFN-γ–producing T cells appeared unchanged (Fig. 1A). The promotion of a Th17 phenotype in LoD cultures was confirmed by the increased secretion of IL-17 in restimulated LoD cells (Fig. 1B) and by the enhanced expression of RORc at day 6 of culture (Fig. 1C). Furthermore, LoD cells secreted significantly lower levels of IFN-γ (Fig. 1B). Thus, although the proportion of IFN-γ–producing cells was not significantly affected by cell density, the amount of IFN-γ produced was (Fig. 1A versus Fig. 1B). The pro-Th17 effect of low cell density also was observed when T cells were activated by allogeneic DC instead of CD3/CD28 beads; culturing cells under LoD conditions favored Th17 responses (Fig. 1D). The enhancement of Th17 cells in LoD cultures was evident in proportional, as well as absolute, terms; the absolute number of IL-17–producing T cells was significantly higher in LoD cultures than in HiD cultures (Fig. 1E). The Th17-promoting effect of LoD stimulation required the presence of pro-Th17 cytokines: higher percentages of IL-17 producers were observed in LoD cultures supplemented with pro-Th17 cytokines (Fig. 1F, left panel) but not in cultures in which pro-Th17 cytokines had not been added ("monopolarizing," Fig. 1F, middle panel). Nonetheless, even a reduction in the concentration of pro-Th17 cytokines in LoD cultures to a level similar to that in HiD cultures on a per-cell basis efficiently promoted Th17 responses, suggest-
ing that impaired Th17 responses under HiD conditions could not be explained by insufficient availability of pro-Th17 cytokines (data not shown). Furthermore, LoD stimulation did not enhance Th1 responses in the presence of the pro-Th1 cytokine IL-12, indicating that the effect of cell density is selective for Th17, but not for Th1, responses (Fig. 1F, right panel). We showed previously that low-strength T cell activation promotes Th17 cells (18). The pro-Th17 effect of LoD cultures could not be explained by a lower strength of T cell stimulation, because activation of T cells in both HiD and LoD cultures effectively induced the expression of OX40 ligand (Supplemental Fig. 1) and CD25 (data not shown) in all T cells, unlike suboptimal T cell activation conditions with a low T cell/bead ratio. In addition, no differences were observed in the proliferative capability (Supplemental Fig. 2) or cell viability (Supplemental Fig. 3) of T cells in HiD and LoD cultures. Taken together, these data show that the cell density of memory CD4+ T cells during their activation determines their ability to generate a Th17 response.

Slower kinetics of STAT3 activation in LoD T cells compared with HiD T cells

Because the transcription factor STAT3 plays a central role in the induction of Th17 responses, we addressed the question of whether STAT3 activation was impaired in HiD cultures, explaining their poor ability to generate a Th17 response. Surprisingly, Western blot experiments showed that, after 24 h of activation, HiD cells
exhibited high levels of tyrosine phosphorylated STAT3 (STAT3-pY705) in both the absence and presence of pro-Th17 cytokines (Fig. 2A). In contrast, although activation of LoD cells also induced tyrosine phosphorylation of STAT3, the levels of STAT3-pY705 were slightly lower and required the presence of pro-Th17 cytokines to reach similar levels as observed in HiD cells (Fig. 2A). The levels of total STAT3 protein were similar in HiD and LoD cultures (Fig. 2A). Furthermore, STAT3 serine phosphorylation followed a similar pattern as STAT3-pY705; levels were higher in HiD cells than in LoD cells (data not shown). Thus, these data indicate that STAT3 phosphorylation in HiD cells was not impaired and, particularly in the absence of pro-Th17 cytokines, appeared to be even higher than in LoD cells. To obtain a clearer picture of STAT3 activation in HiD and LoD cultures, STAT3 tyrosine phosphorylation was measured at the single-cell level by flow cytometry over the 6-d culture period.

Interestingly, we found major differences in the STAT3 activation kinetics between HiD and LoD cultures. STAT3 in HiD cultures became rapidly (within 24 h) phosphorylated in a large proportion of the cells, whereas STAT3 phosphorylation in LoD cultures was much slower, taking 72 h before STAT3-pY705 levels were similar to HiD cultures (Fig. 2B). In contrast, the activation kinetics of STAT1 during the first 48 h were more similar between HiD and LoD cultures (Supplemental Fig. 4), possibly explaining why cell density does not affect IFN-γ responses. STAT3 activation in HiD cells during the first 72 h was independent of exogenously added pro-Th17 cytokines; however, for STAT3 activation to be sustained for 6 d, pro-Th17 cytokines were required (Fig. 2B). The presence of pro-Th17 cytokines in LoD cultures did not accelerate the slow STAT3 activation kinetics in LoD cells (Fig. 2B), but it did appear to enhance the number of STAT3-pY705+ cells during the first 72 h of culture, although this enhancement was not statistically significant (Fig. 2B). As observed for HiD cells, LoD cells also required pro-Th17 cytokines to maintain STAT3-pY705 levels; in the absence of these exogenous cytokines, STAT3-pY705 levels dropped sharply after 72 h of activation. Furthermore, the rapid and high STAT3 activation in HiD cells is most likely due to autocrine or paracrine signaling through endogenously produced cytokines, because the JAK1/2 inhibitor ruxolitinib reduced STAT3-pY705 levels in HiD cultures (Fig. 2C).

Taken together, these data show that STAT3 activation per se in HiD T cells is not impaired and that these cells remain responsive to STAT3-activating signals throughout the 6-d culture.

Inhibition of early STAT3 activation in HiD T cells promotes Th17 responses

Because the most striking difference between HiD and LoD T cells was the rapid versus slow kinetics of STAT3 activation, respectively, we hypothesized that early STAT3 activation in HiD T cells induces a negative-feedback loop that interferes with the formation of IL-17–producing T cells. Using a small chemical inhibitor of STAT3 phosphorylation, S3I-201, the rapid and high level of STAT3 activation during the first 48 h of HiD T cell activation

**FIGURE 2.** Early constitutive STAT3 activation in HiD cultured cells. (A) Unstimulated, HiD and LoD human CD4+CD45RO+ T cells were cultured in the absence (-) or presence (+) of pro-Th17 cytokines for 24 h. Whole-cell lysates were prepared, and STAT3 expression and phosphorylation of tyrosine 705 (STAT3-pY705) were determined by Western blotting. Blot is representative of two independent experiments. (B) Human CD4+CD45RO+ T cells were cultured under HiD and LoD conditions in the absence (-) or presence (+) of pro-Th17 cytokines for 24, 48, 72, 96, 120, or 144 h. Expression of STAT3-pY705 was determined by intracellular staining. Representative 24-h HiD and LoD plots in the absence or presence of pro-Th17 cytokines (upper panels). Percentages of STAT3-pY705+ cells are shown as mean ± SEM (n = 3) (lower panels). *p < 0.05, **p < 0.01, and ***p < 0.001, two-way ANOVA. (C) Human CD4+CD45RO+ T cells were cultured under HiD conditions with pro-Th17 cytokines, in the absence or presence of the JAK1/2 inhibitor ruxolitinib, and expression of STAT3-pY705 was determined by intracellular staining at 24 h. Data are mean ± SEM (n = 3). *p < 0.05, paired t test.
could be inhibited; after this time point STAT3-pY705 levels gradually increased (Fig. 3A). Thus, using the STAT3 inhibitor in HiD cultures resulted in STAT3 activation kinetics that were quite similar to the STAT3 kinetics of LoD cultures (Fig. 2B). Interestingly, slowing down the kinetics of STAT3 activation in HiD cultures significantly enhanced the generation of Th17 responses (Fig. 3B), suggesting that a STAT3-mediated mechanism is responsible for the impaired Th17 responses in HiD cultures.

**Impaired Th17 responses in HiD T cells is not due to STAT3-regulated cytokine production**

We considered the possibility that inhibition of STAT3 reduced the secretion of STAT3-regulated cytokines that play a role in controlling Th17 polarization in an autocrine or paracrine manner. Indeed, STAT3 inhibition strongly and significantly inhibited secretion of IL-6 and IL-10 in HiD cultures; levels of these cytokines in STAT3-inhibited HiD cultures dropped to levels comparable to LoD cultures (Fig. 4A). In contrast, secretion of TNFα and IFN-γ was not significantly reduced by STAT3 inhibition (Fig. 4A). We next investigated whether the reduction in IL-6 or IL-10 secretion was responsible for the beneficial effect of STAT3 inhibition in HiD cultures. However, blocking of IL-6R or IL-10R in HiD cultures did not enhance the generation of Th17 cells (Fig. 4B). We next assessed the possibility that a reduction in STAT3 phosphorylation inhibited the secretion of another (known or unknown) Th17-inhibitory soluble factor. In an attempt to remove putative inhibitory factors from HiD cultures, cells were washed 24, 48, or 72 h after activation and recultured in the presence of pro-Th17 cytokines. However, washing of HiD cells did not enhance their ability to mount a Th17 response (Fig. 4C). Furthermore, addition of conditioned media from HiD cultures to LoD cultures did not reduce Th17 responses in LoD cultures (Fig. 4D). Together, these data suggest that impaired Th17 responses in HiD cultures cannot be explained by the autocrine-negative action of a STAT3-regulated cytokine.

**High ectonucleotidase activity in HiD cultures inhibits Th17 responses**

Because our data argue against a role for inhibitory soluble factor(s) in impairing Th17 responses, we considered the possibility that membrane-bound molecule(s) were involved. In keeping with this notion, disruption of T cell clusters by regular pipetting during the first 3 d of HiD culture significantly enhanced IL-17–producing, but not IFN-γ–producing, T cells (Fig. 5A), suggesting that a cell contact–dependent mechanism inhibited Th17 cells. Recently, it was reported that the ectonucleotidase CD39 (which catalyzes hydrolysis of ATP and ADP into AMP) is expressed by a subset of regulatory T cells (Tregs) and that these Tregs inhibited IL-17 production through CD39-mediated ectonucleotidase activity (31). Because expression of CD39 is regulated by STAT3 (32), we addressed the question of whether enhanced expression of CD39 in HiD T cell cultures was responsible for limiting Th17 responses. HiD T cells expressed significantly higher levels of CD39 than did LoD T cells (Fig. 5B), and the low IL-17 production by HiD cells was enhanced by the CD39 inhibitor POM-1 (Fig. 5C). Conversely, the high IL-17 response in LoD T cell cultures could be inhibited by a P2X7 receptor (ATP receptor) antagonist. Taken together, these results suggest that the high ectonucleotidase activity of HiD T cells limits their ability to produce IL-17 and indicate that ATP is required for optimal Th17 responses.

**Discussion**

Over the last decade, evidence has mounted that Th17 cells play a role in the pathogenesis of chronic inflammatory diseases, including autoimmune diseases. Therefore, understanding how these cells are regulated has become a major research question. In this study, we demonstrate that a rapid and strong activation of STAT3, as observed under high T cell density conditions, inhibited Th17 responses. Slowing down of STAT3 activation by using a STAT3 inhibitor or by activating T cells under low cell density conditions promoted Th17 responses, indicating that the kinetics of STAT3 activation plays a key role in determining the IL-17–producing ability of human memory T cells. The STAT3-mediated negative-feedback loop was associated with enhanced CD39 expression on HiD cells. Inhibition of CD39 ectonucleotidase activity restored Th17 responses under HiD conditions, whereas, conversely, inhibition of the P2X7 ATP receptor inhibited Th17 cells in LoD cultures. Thus, our data also indicate that ATP plays a crucial and direct role in regulating the IL-17–producing capacity of memory T cells.

STAT3 undoubtedly plays an important role in the induction and maintenance of Th17 responses. STAT3 is activated by a number of pro-Th17 cytokines (e.g., IL-6, IL-23, and IL-21), and it is involved in the activation of a number of Th17 response genes; mutations in STAT3 can cause Th17 deficiency in humans, and T cell–targeted STAT3 deletion completely inhibits Th17 development in mice (22–24, 33). However, STAT3 also can induce expression of proteins that are inhibitory to Th17 cells [i.e., suppressor of cytokine signaling-3 (SOCS3) (34)]. This negative-feedback regulator of STAT3 acts by specifically inhibiting the action of JAK tyrosine kinases at certain cytokine receptors. In mice, SOCS3 inhibits the differentiation of IL-17–secreting T cells by impairing IL-23–mediated STAT3 phosphorylation (35). In our model, it is unlikely that SOCS3 was responsible for the impaired formation of IL-17–producing T cells in HiD cultures, because SOCS3 protein levels in T cells 24 h after activation were, in fact, lower in HiD cultures than in LoD cultures (data not shown); IL-23 effectively induced STAT3 tyrosine phosphorylation in both HiD and LoD cells harvested at the 72-h time point (data not shown);
and STAT3 tyrosine phosphorylation levels remained high until the end of culture (i.e., day 6) in both HiD and LoD cultures after the addition of IL-23 on day 3 (Fig. 2B). Thus, IL-23 responsiveness was not impaired in HiD cells.

Interestingly, in contrast to previous studies showing an inhibitory role for SOCS3 in Th17 differentiation (35–37), a recent study by Kleinsteuber et al. (38) found that overexpression of SOCS3 in human memory T cells promotes IL-17 production. A possible explanation for this discrepancy is that the role of SOCS3 is different in Th17 polarization versus activation of memory T cells that may be precommitted to a Th17 phenotype. Although, the study by Kleinsteuber et al. (38) did not directly address whether the Th17-promoting effect of SOCS3 was mediated through a reduction in STAT3 activation, we consider our findings to be in line with theirs in that we identified a Th17-inhibitory role for the STAT3-signaling pathway within a memory T cell population.

In this study, we did not address whether the enhanced numbers of Th17 cells after STAT3 inhibition were due to enhanced expansion of precommitted Th17 cells or de novo induction of Th17 cells from uncommitted memory T cells. However, enriching the memory T cell population for CCR6 expression, a chemokine receptor selectively expressed by Th17 cells (39), markedly enhanced the number of Th17 cells under both HiD and LoD conditions, suggesting that the former is the case (data not shown). We also did not specifically address which endogenously produced cytokines were involved in the rapid STAT3 kinetics, but it is likely that a combination of STAT3-inducing cytokines was involved (e.g., IL-6, IL-10, LIF, IL-21). Further studies are required to address these questions.

Our data do not repudiate the notion that STAT3 is critically required for the induction of Th17 responses, but they do provide a nuance to the regulatory role of this transcription factor in that it can have a Th17-inhibitory function in memory T cells. The STAT3-signaling pathway has been proposed as a potential target for therapeutic intervention in autoimmunity, chronic inflammatory diseases, and cancer (34, 40, 41). In this respect, our findings are likely to have relevance because inhibition of STAT3 may actually enhance the number of IL-17–secreting T cells within memory T cell populations—an effect that may not be the desired outcome of STAT3-targeting therapies.

Our data suggest that the mechanism by which STAT3 limits IL-17–producing T cells in memory T cell cultures involves the STAT3-regulated ectonucleotidase CD39, which converts ATP and ADP into AMP. Because STAT3 was shown to bind to the CD39 promoter and enhance its expression (32), we propose that the enhanced STAT3 activation in HiD cells is responsible, at least in part, for the higher expression levels of CD39 in these cells compared with LoD cells. The high CD39 expression by HiD cells explains why IL-17 production in these cells is limited; inhibition of CD39 ectonucleotidase activity restored Th17 responses under HiD conditions (Fig. 3C). CD39 was reported to be involved in the

**FIGURE 4.** Impaired Th17 response in HiD T cells is not due to STAT3-regulated cytokine production. (A) Human CD4+CD45RO+ T cells were activated with anti-CD3/CD28 beads in the presence of pro-Th17 cell cytokines and cultured under LoD conditions or HiD conditions in the absence or presence of the STAT3 inhibitor S31-201. At 48 h, cytokine secretion was determined by ELISA. Data are mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, two-way ANOVA. (B) Human CD4+CD45RO+ T cells were activated with anti-CD3/CD28 beads in the presence of pro-Th17 cell cytokines and cultured under LoD or HiD conditions in the absence or presence of anti–IL-10R, anti–IL-6R, or isotype-matched controls for 6 d. Expression of intracellular IL-17 was determined by flow cytometry following 5 h of PMA/ionomycin restimulation. Data are mean ± SEM (n = 3). (C) Human CD4+CD45RO+ T cells were activated with anti-CD3/CD28 beads in the presence of pro-Th17 cell cytokines and cultured under HiD conditions. After 24, 48, or 72 h, cells were harvested, washed, and replated in fresh medium containing pro-Th17 cytokines. Expression of intracellular IL-17 was determined at day 6 by flow cytometry following 5 h of PMA/ionomycin restimulation. Data are mean ± SEM (n = 3). (D) Cell-free conditioned media were obtained from 24- and 72-h HiD cultures. CD4+CD45RO+ T cells were activated with anti-CD3/CD28 beads in the presence of pro-Th17 cell cytokines and cultured under LoD conditions in the presence of 50% HiD conditioned media. Expression of intracellular IL-17 was determined at day 6 by flow cytometry following 5 h of PMA/ionomycin restimulation. Data are mean ± SEM (n = 3).
function and regulation of Th17 cells. CD39 can be expressed by Th17 cells themselves, and these CD39-expressing Th17 cells were shown to have immunosuppressive function (32). Furthermore, the active form of vitamin D3 was shown to suppress IL-17-producing T cells through upregulation of CD39 expression (42). It was reported that CD39 is expressed by a subset of murine Tregs and that these Tregs constrain Th17 cells through a combined action of CD39 and CD73, with CD39 converting ATP and ADP into AMP and CD73 further cleaving AMP into adenosine (31). Although we did not explore the possibility that adenosine impaired Th17 responses in HiD cultures, we do not think that it is the most likely scenario, because adenosine is known to strongly inhibit IFN-γ production (31, 43); we did not observe any significant differences between HiD and LoD cultures in terms of their IFN-γ-producing potential (Fig. 1). Furthermore, inhibition of the purinergic P2X7 receptor inhibited IL-17-producing, but not IFN-γ-producing, T cells in LoD cultures (Fig. 5). Thus, taken together, our data indicate that extracellular ATP plays a crucial role in regulating the IL-17-producing capacity of memory T cells.

It was proposed that the release of ATP by immune cells, including activated T cells, provides an important autocrine feedback loop that modulates immune cell function (reviewed in Ref. 44). For example, extracellular ATP inhibits IL-12 and IL-27 production but enhances IL-23 secretion in moDCs (45). Interestingly, ATP was reported to drive Th17 responses in an indirect manner, through induction of pro-Th17 cytokines in DCs (46–48). In this study, we demonstrated for the first time, to our knowledge, that ATP is also required for the optimal formation of IL-17-producing cells in a memory T cell population, thus suggesting a direct effect on T cells themselves. Notably, the promotion of Th17 cells required the presence of pro-Th17 cytokines (Fig. 1D). Thus, ATP appears to act at different levels: at the DC level it enhances the secretion of pro-Th17 cytokines, and at the T cell level it promotes Th17 responses within a memory T cell population.

This study originated from our initial observation that the cell density during activation of memory T cells determines the extent of the Th17 response. The use of an in vitro model to investigate effects of cell density on the nature of the immune response constitutes a major limitation of our study, because results are difficult to extrapolate to the in vivo situation. Nevertheless, the cell density model has been instrumental in revealing a STAT3-mediated negative-feedback loop limiting Th17 responses. Furthermore, our data may be informative for other investigators conducting in vitro studies to delineate mechanisms controlling Th17 responses.

In conclusion, we showed that memory Th17 responses can be inhibited through a STAT3-dependent mechanism. Our results suggest that STAT3 acts through enhancing the CD39 ectonucleotidase activity of memory T cells. Therefore, our data also indicate a direct role for ATP in promoting a Th17 response from memory T cells. Because inflammation and tissue damage result in the release of ATP, which is now regarded as a “danger signal” with proinflammatory properties, the enhanced ectonucleotidase activity of memory T cells under high cell density conditions may serve as a safety mechanism by which Th17 cell formation can be limited during overly exuberant immune responses. Our results may also explain why under certain chronic inflammatory conditions that are thought to be driven by Th17 cells, such as autoimmune arthritis [e.g., RA (49) or juvenile arthritis (50)], Th17 cells are difficult to find in the chronically inflamed tissue (51). Indeed, synovial T cells from juvenile arthritis patients have enhanced expression of CD39, possibly explaining why the numbers of IL-17-producing synovial T cells are very low, whereas IFN-γ-producing T cells are readily detectable (52). Finally, our results have relevance in relation to the development of small molecule inhibitors of the STAT3-signaling pathway for the treatment of autoimmune disease; if suppression of Th17 responses is the desired goal, care should be taken because inhibition of STAT3 may,
INHIBITION OF Th17 THROUGH STAT3


