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A Negative Feedback Loop Mediated by STAT3 Limits Human Th17 Responses

Harriet A. Purvis, Amy E. Anderson, David A. Young, John D. Isaacs, and Catharien M. U. Hilkens

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 pool. 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.

The helper 17 cells are a subset of CD4+ Th cells that are defined predominantly by the production of their signature cytokine IL-17A, hereafter referred to as IL-17 (1, 2). In addition, Th17 cells are characterized by secretion of IL-17F, IL-21, and IL-22, as well as expression of the transcription factor RORγT/RORc (reviewed in Refs. 3–5). Th17 cells play a key role in antimicrobial defense (6); however, dysregulated Th17 cell responses have been implicated in the pathogenesis of certain types of organ-specific graft-versus-host disease [e.g., skin graft-versus-host disease (7, 8)] and autoimmune diseases [e.g., rheumatoid arthritis (RA), psoriasis (9–11)]. Th17 cells contribute to chronic tissue inflammation via a variety of mechanisms, including the recruitment of neutrophils and the induction of additional proinflammatory mediators, including IL-1β, IL-8, and matrix metalloproteinases 1 and 13 (12, 13).

Because Th17 cells can play a pathogenic role in a number of chronic inflammatory diseases, it is important to understand how these cells are regulated. The cytokine milieu in which T cells encounter their cognate Ag is instrumental to the polarization of Th17 responses. Combinations of IL-1β, and/or IL-6, and/or TGF-β were shown to induce Th17 cell differentiation, whereas IL-23 expands and stabilizes established Th17 cells (reviewed in Refs. 3, 4). In addition to the cytokine milieu, other factors have recently emerged as potent regulators of Th17 cells, including NaCl (14, 15) and NO (16, 17). Furthermore, we have shown previously that human Th17 cell responses are promoted by low-strength TCR activation (18).

The combination of TCR signaling and environmental pro-Th17 cytokines activate a number of transcription factors, including NFATc1, RORc, and STAT3, which together allow for IL-17A expression and the development of Th17 cell responses (19–21). The transcription factor STAT3 has a particularly important role in regulating Th17 cells. A dominant negative STAT3 mutation in patients with hyper-IgE syndrome causes a deficiency in Th17 cells that contributes to the development of chronic mucocutaneous candidiasis (22, 23). In addition, the development of pathogenic Th17 cell responses, causing colitis/inflammatory bowel disease, is dependent on STAT3 (24). In RA patients, STAT3 expression positively correlates with synovitis and enhances the Th17-differentiation potential of RA synovial T cells (25). Chromatin immunoprecipitation sequencing studies revealed that STAT3 directly regulates a number of Th17 cell response genes, including RORC, RORa, IL-17F, IL-21, and IL-17A itself, explaining its critical role in promoting Th17 responses (24).

A number of studies in the cancer field demonstrated that cell confluence/density can have a profound effect on STAT3 activation and, thus, transcriptional regulation (26, 27). The consequences of cell density on T cell responses are not often addressed in immune regulation studies, but they may be an important factor to consider. For example, T cell densities differ depending on their location (e.g., lymph node versus peripheral tissues) and on the level and chronicity of inflammation. Therefore, we addressed the question of how cell density affects the STAT3 activation kinetics and ability of human T cells to produce IL-17. In this study, we fo-
cused 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 Committees 2. PBMCs were isolated by 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 were 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 peptidoglycan 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-23 was refreshed on day 3 with the addition of 10 U/ml IL-2 (PeproTech; Novartis). Intracellular cytokine staining was performed as described below. For selected cultures, the STAT3 inhibitor SU-201 (50 μM; Calbiochem) was added to HiD cultures on days 0 and 1, the JAK1/2 inhibitor ruxolitinib (50 μM; Calbiochem) was added on days 0 and 3. CD39 expression was determined on day 6 by flow cytometry using mAb anti-CD39–FITC (clone TU66; BD Pharmingen). Intracellular cytokine staining was performed on a per-cell basis efficiently promoted Th17 responses, suggesting that the proportion of IFN-γ producers was (Fig. 1A versus 1B). The enhancement of Th17 cells in LoD cultures was evident in proportional, as well as absolute, terms; the increase in the percentage of IL-17–producing T cells, whereas the proportion of IFN-γ-producing cells was not significantly affected by cell density, the amount of IFN-γ production was (Fig. 1A). The 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 cultures 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). The Th17 response 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 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.

**FIGURE 1.** HiD culture of memory T cells specifically impairs Th17 cell responses. (A–C, and E) Human CD4+CD45RO+ T cells were activated with anti-CD3/CD28 beads in the presence of pro-Th17 cell cytokines and cultured under HiD and LoD conditions for 6 d. (A) T cells were restimulated with PMA/ionomycin for 5 h, and expression of intracellular IL-17 and IFN-γ was determined by flow cytometry. Plots of 1 representative example of 16 experiments (left panels) and percentages of IL-17 and IFN-γ producers from 16 independent experiments (right panels). Horizontal bars represent median value. ***p < 0.01, paired t test. (B) T cells were harvested, washed, and replated at 1 × 10^6 cells/ml and restimulated with PMA/ionomycin for 24 h. IL-17 and IFN-γ levels in supernatants were determined by ELISA. Data are mean ± SEM (n = 3). *p < 0.05, **p < 0.01, paired t test. (C) T cells were harvested at 0, 48, 96, or 144 h, and expression of RORc was determined by real-time PCR and normalized to RORc expression at 0 h. Data are mean ± SEM (n = 3). *p < 0.05, two-way ANOVA. (D) Human CD4+CD45RO+ T cells were cultured for 6 d with peptidoglycan/C. albicans-activated autologous moDCs at a 1:10 moDC/T cell ratio under either HiD or LoD conditions. Expression of IL-17 and IFN-γ was determined by intracellular flow cytometry. Data are mean ± SEM (n = 3). *p < 0.05, two-way ANOVA. (E) The number of live cells on day 6 was determined by cell counting using trypan blue exclusion, and the absolute number of IL-17+ and IFNγ+ cells was calculated using the proportion of IL-17 or IFN-γ cells, as determined by flow cytometry (n = 3). *p < 0.05, paired t test. (F) Day 6 expression of intracellular IL-17 and IFN-γ from HiD and LoD cells cultured in the presence of pro-Th17 cytokines (left panel), nonpolarizing cytokines (middle panel), or pro-Th1 cytokines (right panel) (n = 4). Data are mean ± SEM. ***p < 0.001, two-way ANOVA.
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, S31-201, the rapid and high level of STAT3 activation during the first 48 h of HiD T cell 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).

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**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 three 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 < 0.001, two-way ANOVA. (C) Human CD4+CD45RO+ T cells were cultured under HiD conditions with or without 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 respon-
siveness was not impaired in HiD cells.

Interestingly, in contrast to previous studies showing an inhib-
itory 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 pop-
ulation.

In this study, we did not address whether the enhanced numbers
of Th17 cells after STAT3 inhibition were due to enhanced ex-
pansion 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 en-
hanced the number of Th17 cells under both HiD and LoD con-
ditions, 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 in-
volved (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. 5C). 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 after the
addition of IL-23 on day 3 (Fig. 2B). Thus, IL-23 respons-
siveness was not impaired in HiD cells.

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explains why IL-17 production in these cells is limited; inhibition
of CD39 ectonucleotidase activity restored Th17 responses under
HiD conditions (Fig. 5C). CD39 was reported to be involved in the
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 signif-
icant 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, in-
cluding 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 produc-
tion 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 re-
quired 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. Fur-
thermore, 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 ectonucle-
otidease 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 activ-
ity 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 con-
ditions that are thought to be driven by Th17 cells, such as au-
toimmune 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 en-
hanced 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 de-
sired goal, care should be taken because inhibition of STAT3 may,
in certain microenvironments, enhance the formation of IL-17-producing memory T cells.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. LoD and TLo conditions have different activation kinetics. Memory CD4+ T-cells cultured under either HiD, LoD or low T-cell stimulation conditions (1 bead: 50 T-cells – TLo) conditions. At the start of culture (Day 0) and 24 (A) or 72 (B) hours, cell surface expression of OX40 was determined by flow cytometry, (n=3); error bars represent SEM, p-values calculated by one-way ANOVA *<0.05 **<0.01, ***<0.001.

Supplemental Figure 2. No difference in HiD and LoD CD4+ T-cell proliferation. CD4+ T-cells were CFSE labelled and cultured under either HiD or LoD conditions for 6 days. CFSE dilution was determined by flow cytometry. A representative experiment is shown (n=2).
Supplemental Figure 3. No difference in HiD and LoD CD4+ T-cell viability. CD4+ T-cells were cultured under either HiD or LoD conditions for 6 days. Cell viability was determined by Viaprobe staining and measured by flow cytometry. A representative experiment is shown (n=4).

Supplemental Figure 4. Kinetics of STAT1 activation. Human CD4+CD45RO+ T-cells were cultured under HiD or LoD conditions in the absence (left) or presence (right) of proTh17 cytokines for 24, 48, 72, 96, 120, and 144 hours. Expression of STAT1pY701 was determined by intracellular staining (n=2) error bars represent SEM, p-values calculated by two-way ANOVA *<0.05