CD4 T cell-intrinsic role for the T helper 17 signature cytokine IL-17: Effector resistance to immune suppression

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Untoward effector CD4+ T cell responses are kept in check by immune regulatory mechanisms mediated by CD4+ and CD8+ T cells. CD4+ T helper 17 (Th17) cells, characterized by IL-17 production, play important roles in the pathogenesis of autoimmune diseases (such as arthritis, multiple sclerosis, psoriasis, inflammatory bowel disease, among others) and in the host response to infection and cancer. Here, we demonstrate that human CD4+ T cells cells exposed to a Th17-differentiating milieu are significantly more resistant to immune suppression by CD8+ T cells compared to control Th0 cells. This resistance is mediated, in part, through the action of IL-17A, IL-17F, and IL-17AF heterodimer through their receptors (IL-17RA and IL-17RC) on CD4+ T cells themselves, but not through their action on CD8+ T cells or APC. We further show that IL-17 can directly act on non-Th17 effector CD4+ T cells to induce suppressive resistance, and this resistance can be reversed by blockade of IL-1β, IL-6, or STAT3. These studies reveal a role for IL-17 cytokines in mediating CD4-intrinsic immune resistance. The pathways induced in this process may serve as a critical target for future investigation and immunotherapeutic intervention.

immune regulation | effector T cell resistance | CD4 | CD8 | Th17

CD4+ T helper (Th) cell subsets have largely been defined by the cytokines they secrete and more recently by certain key transcription factors expressed. The initial paradigm of IFNγ-producing Th1 cells and IL-4–producing Th2 cells (1, 2) has been a useful framework in the understanding of Th differentiation and function. Th17 cells, characterized by their production of IL-17, are a more recently defined subset (3, 4) that helped explain several observations not clearly resolved through the Th1/Th2 paradigm. Th17 cells play critical roles in the body's response to infections and cancer and in the pathogenesis of autoimmune diseases (3, 5–8). At the same time, there are important regulatory immune mechanisms in place to keep CD4+ T cell responses in check, such as regulatory/suppressor CD4+ and CD8+ T cells (9–14).

Effector T cell resistance to such suppressive mechanisms may play a role in both the immune response to infection/cancer and in mediating autoimmunity. Indeed, CD4+ effector T cells have been shown to be differentially resistant to suppression by CD4+ T regulatory cells (Tregs) and CD8+ T cells, as reported by us and others in the setting of various autoimmune diseases, such as multiple sclerosis, type 1 diabetes, and arthritis (12, 13, 15–17).

In the current study, we investigated whether suppressive resistance might be a feature of specific Th subtypes. We demonstrate that human CD4 T cells exposed to Th17-differentiating conditions were greatly resistant to suppression compared to Th cells grown in media alone (control Th0 cells). In contrast, Th1 cells were significantly more sensitive to suppression. We uncovered a function for Th17-secreted IL-17 cytokines in mediating Th resistance and the mechanistic pathways involved in this autocrine/paracrine process, which have broad implications for intensive investigation and therapeutic intervention in the setting of immune-mediated diseases.

Results and Discussion

Th17 Differentiation Results in Resistance to Immune Suppression. A deficit of immune regulation is implicated in the causation and progression of autoimmunity. Recently, it has been appreciated that this "regulatory deficit" may be a reflection of heightened resistance in effector T cells to suppressive mechanisms (13, 18–21). We hypothesized that different lineages of effector CD4+ T cells may have differential resistance to suppression. To address this hypothesis, we obtained highly enriched populations of naïve CD4+ T cells from healthy donor peripheral blood mononuclear cells (PBMCs) and cultured them in vitro under the influence of different cytokine combinations to stimulate their differentiation along various T helper pathways (*SI Appendix*, Fig. S1), using conditions described in previously published studies (22–24). At day 7 of culture, we confirmed that the differentiated populations of cells exhibited the expected functional phenotypes in terms of cytokine production. In particular, we confirmed that the Th0 control condition and the Th1 condition showed predominantly IFNγ production but almost undetectable IL-17A, whereas the Th17 conditions resulted in minimal IFNγ but robust IL-17A production (*SI Appendix*, Fig. S2*A*), in keeping with previously published observations (23, 24). As there was anti-IFNγ added to some of the cultures that might interfere with quantification, we also confirmed the IFNγ pattern using intracellular cytokine staining by flow cytometry (*SI Appendix*, Fig. S2*B*). Similarly, since IL-4 was added to some of the cultures, we measured multiple surrogate Th2 cytokines like IL-5, IL-10, and IL-13 (*SI Appendix*, Fig. S2*A*).

Significance

CD4+ T helper (Th) cells come in several flavors, largely defined by their cytokine profiles. Th17 cells, characterized by the production of IL-17 family cytokines, play important roles in the body's response to infections and cancer and in the pathogenesis of autoimmune diseases. This study reveals an unexpected role for the T helper 17 signature cytokines: IL-17A, IL-17F, and IL-17AF. We show that these cytokines can act directly on CD4 T cells to make them resistant to immune regulation. This resistance is mediated through IL-1β–mediated and IL-6–mediated pathways in a T cell-intrinsic manner. These findings have broad implications in multiple settings involving immune-mediated responses (including autoimmunity, cancer therapy, and chronic infectious disease).

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The authors declare no competing interest.

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Collectively, these data showed that the differentiation cultures resulted in the expected overall cytokine patterns.

We then tested whether these CD4+ T cells showed a differential ability to be suppressed. For this, we utilized an in vitro flow cytometric suppression assay system established by us previously (12, 13) to assess suppression of responder CD4+ T cells by bulk ex vivo CD8+ T cells. Thus, cells from the various CD4+ T cell differentiation cultures were used as responder T cells in flow cytometric suppression assays. The cells were stimulated with autologous APCs and αCD3 and cultured in the presence (or absence) of bulk autologous CD8+ T cells, which are known to robustly suppress CD4+ proliferation (12). On day 7 of these suppression cultures, the proliferation of CD4+ T cells was quantified (based on CFSE dilution) and then normalized to the 1:0 "no suppression" control condition (no CD8+ T cells). We observed that the various Th-cell lineages showed comparable proliferative capacity in the absence of CD8+ T cells (Fig. 1*A* and *SI Appendix*, Fig. S3*A*). However, they underwent different levels of suppression by CD8+ T cells. Compared to the Th0 control cells, Th1 cells showed a significantly greater susceptibility to suppression (Fig. 1*B*), whereas suppression of Th2 cells was not significantly different from that of Th0 cells. In contrast, Th17 cells showed a remarkably high resistance to suppression with significantly lower %suppression compared to the Th0 controls (Fig. 1 *B* and *C*). Representative raw proliferation data are demonstrated in *SI Appendix*, Fig. S3*A*. In addition to measuring proliferation, we also quantified CD25 expression on these cells, representing activation. Matching with our previous observations from this assay system, the CD25 expression pattern matched the proliferation pattern, in that there were no differences in CD25 expression in the various 1:0 conditions, whereas in the presence of CD8+ T cells, the CD25 expression was similar in Th0 and Th2 conditions, significantly suppressed in the Th1 condition and significantly preserved in the Th17 condition (*SI Appendix*, Fig. S3*B*). Quantification of apoptosis did not reveal significant differences across the cultures whether measured at day 7 of differentiation or within the suppression cultures with or without CD8+ T cells (*SI Appendix*, Fig. S3*C*). Collectively, these results suggested that while cells from the Th1 cultures were easier to suppress, those from the Th17 cultures had an intrinsic resistance to suppression.

CD4+ T Cell Resistance Is Mediated by T Cell-Derived IL-17. Th17 cells are characterized by their production of certain signature cytokines, such as IL17-A, IL-17F, IL-17AF heterodimer, IL-21, and IL-22 (25, 26). So, we asked whether any of these cytokines may be the mediators of their resistance to suppression. We obtained differentiated cultures of Th0 control cells as well as Th17 cells and then subjected them to suppression assays in the presence or absence of antibodies targeted against the different cytokines. We observed that the Th0 control cells were suppressed equally well in all of the conditions (Fig. 2*A* and *SI Appendix*, Fig. S4*A*). This was an expected result as Th0 cells do not produce any of these cytokines in appreciable quantities.

In contrast, when neutralizing antibodies to IL-17 were added to the Th17 suppression cultures, they significantly reversed the resistance of these cells to suppression (Fig. 2*B*). This reversal was observed when using anti-IL-17A or anti-IL-17F antibodies, alone or in combination, and also using an antibody against the IL-17AF heterodimer. In contrast, blockade of IL-21 or IL-22 did not result in significant change in the suppressive resistance of Th17 cells (*SI Appendix*, Fig. S4*B*).

IL-17 Imparts Increased Resistance by Direct Action on CD4+ T Cells and Not through Action on CD8+ T Cells or APCs. We next asked whether IL-17 was mediating CD4+ T cell resistance by acting on the CD4+ T cells themselves or indirectly through its actions on CD8+ T cells or APC populations. It is known that IL-17A homodimer, IL-17F homodimer, and IL-17AF heterodimer act through the homodimerized/heterodimerized receptor complex of IL-17RA and IL-17RC (27, 28). Therefore, as a first step, we assessed the expression of two well-known IL-17 receptors, IL-17RA and IL-17RC, on various immune cell types (CD4+ and CD8+ T cells as well as CD19+ B cells and CD14+ monocytes). Uniformly across various immune populations, we observed that the vast majority of cells expressed the IL-17RA receptor, which was expressed on ∼100% of the monocytes and ∼80% of the other cell types (*SI Appendix*, Fig. S5). In contrast, IL-17RC was expressed only on a small subset of cells (∼40% of monocytes and ∼20% of other cell types; *SI Appendix*, Fig. S5), similar to previously published observations (29).

We then incubated cells from differentiated Th17 cultures, bulk CD8+ T cells or APCs with antibodies against IL-17RA, IL-17RC, or a combination of both. Cells were then washed and placed in suppression assays. As seen in Fig. 2*C*, when these receptors were blocked on CD4+ cells from the Th17 cultures, there was a significant reversal of their suppressive resistance. In particular, blockade of both receptors resulted in a complete

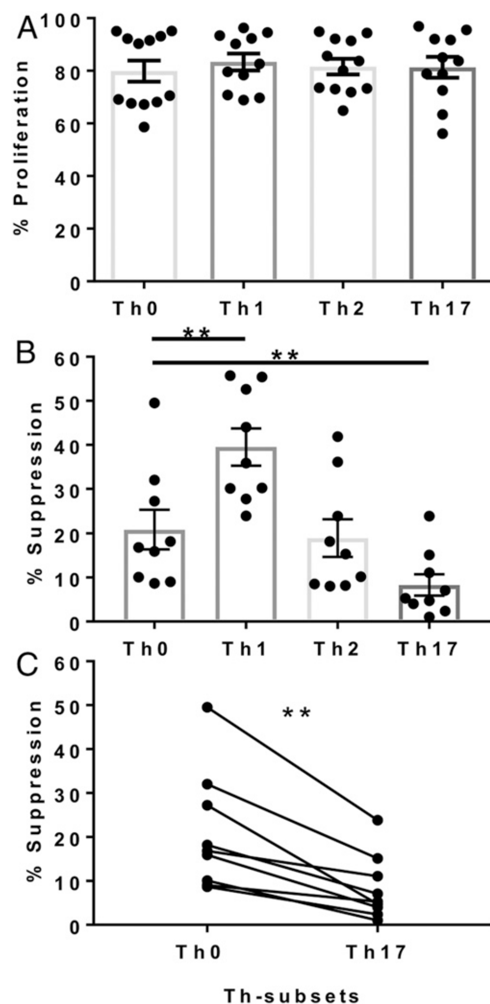

Fig. 1. Th17 cells show greater resistance to CD8 T cell-mediated suppression. Naïve CD4+CD25- T cells obtained from healthy donor PBMCs were polarized under indicated differentiation conditions. On day 7 of cultures, cells were washed twice and stained with CFSE, followed by a 7-d culture with autologous irradiated APCs, fixed αCD3 antibody, and with or without CD8+ T cells. (*A*) Column bars depict %proliferation (±SEM) of indicated Th subsets at a 1:0 ratio (no CD8+ T cells), showing comparable proliferative abilities. (*B*) Column bars depict %suppression (±SEM) of indicated Th subsets by CD8+ T cells at 1:0.5 ratios (CD4+:CD8+). (*C*) Paired %suppression data from the Th0 and Th17 suppression cultures. $^{**}P < 0.005$.

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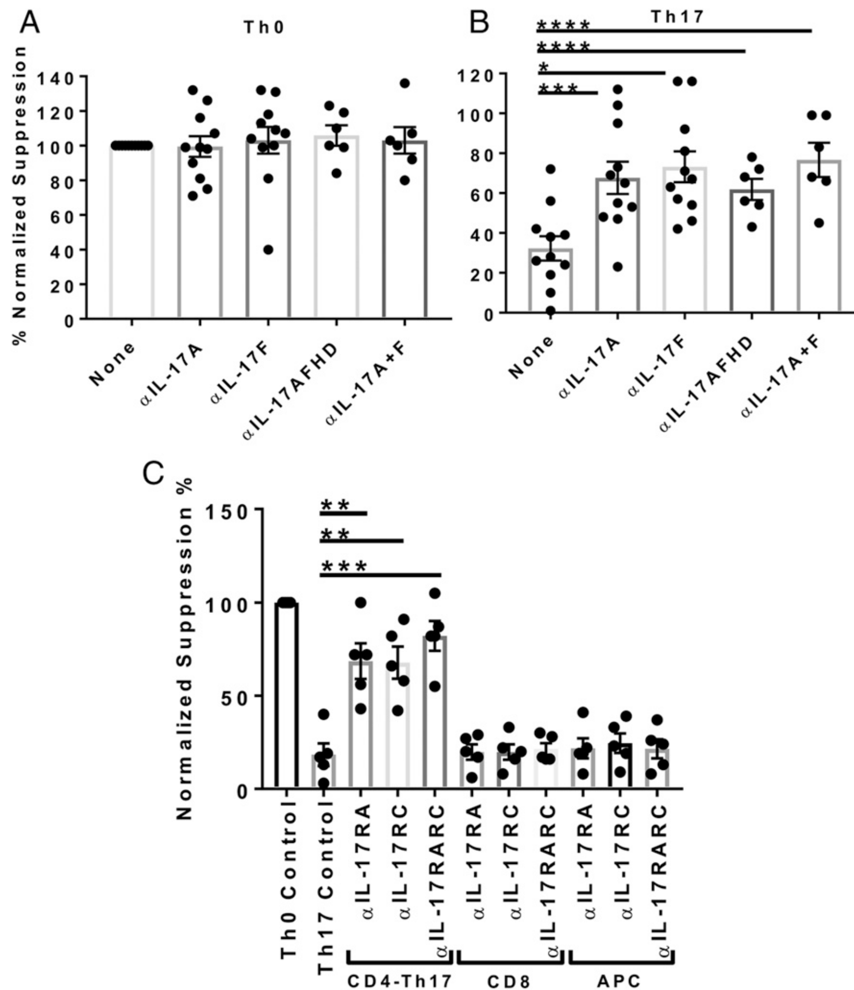

Fig. 2. Reversal of Th17 suppressor resistance following neutralization of IL-17A, IL-17F, or IL-17AF cytokines or blockade of IL-17RA and/or IL-17RC on CD4+ T cells, but not on CD8+ T cells or APC. Naïve CD4+CD25- T cells obtained from healthy donor PBMCs were cultured in Th0 conditions (*A*) or under Th17 differentiation conditions (*B*). Differentiated Th subsets were CFSE-stained and incubated with various IL-17 cytokine neutralizing antibodies, as indicated, and then placed in suppression assays with autologous CD8+ T cells and irradiated APCs and fixed αCD3 for 7 d. (*C*). Th0 and Th17 cells were placed into routine CD8+ suppression assays as in prior figures (Th0 and Th17 controls). In parallel, Th17 cells, autologous APC, or CD8+ T cells were first incubated for 90 min with antibodies against IL-17RA, IL17-RC, or a combination of both. Cells were then washed and used in CD8+ suppression assays in a way that one of the cell types had been preincubated for receptor blockade. The bars indicate normalized suppression data (mean ± SEM), where the baseline suppression observed in the Th0 conditions was designated as 100%. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

reversal, showing suppression that was comparable to the Th0 control cells. Interestingly, preculture blockade of these receptors on either APCs or CD8+ T cells did not have any effect on Th17 resistance (Fig. 2*C*), indicating that IL-17–mediated CD4+ resistance is through a direct autocrine/paracrine action on CD4+ T cells themselves.

Exogenous IL-17 Induces CD4 Resistance to Suppression through IL-1β, IL-6/STAT3 Pathways. Within Th17 differentiation conditions, while there is robust suppression of IFNγ and induction of IL-17, not all CD4+ T cells produce IL-17 by day 7. Several prior reports have shown that a small fraction of the cells might produce this cytokine at that time point (23, 24). At the same time, the resistance to CD8+ suppression is significantly enhanced, with suppression cut by ~50%. Thus, it seemed plausible that the IL-17 secreted by Th17 cells was also acting on CD4+ T cells that are not themselves fully Th17 differentiated. This would be an important distinction since multiple cell types are capable of producing IL-17 in vivo (26). We directly tested this hypothesis by performing suppression assays using otherwise susceptible cells (bulk ex vivo-purified CD4+CD25- T cells) that were first exposed to IL-17A, IL-17F, IL-17AF, or a combination of IL-17A+IL-17F. As expected, these ex vivo-obtained cells were predominantly non-Th17 (0.32% ± 0.07% of CD4+ T cells were IL-17A+). Importantly, IL-17 exposure by itself did not change the baseline proliferative capacity of these cells (Fig. 3*A*), similar to our findings from Fig. 1*A*. However, as seen in Fig. 3 *B–E*, the exposure to IL-17 resulted in greater resistance of these cells to suppression, indicating that exogenously produced IL-17 within the same microenvironment may have the capacity to render neighboring CD4+ T cells resistant to suppression. Interestingly, the IL-17AF heterodimer or a combination of IL1-7A+IL-17F (where the effect could be additive) had the most significant induction of resistance (Fig. 3 *D* and *E*). While the change of suppressive resistance was statistically significant in the case of single cytokines, it did not reach the same magnitude as seen in the Th17 cultures (Figs. 1 and 2), suggesting that other factors may synergize with IL-17A and IL-17F to mediate greater resistance in the context of Th17 differentiation, where other pathways precede the induction of IL-17 production. Taken together, these findings demonstrate a role for IL-17 in imparting CD4 effector resistance.

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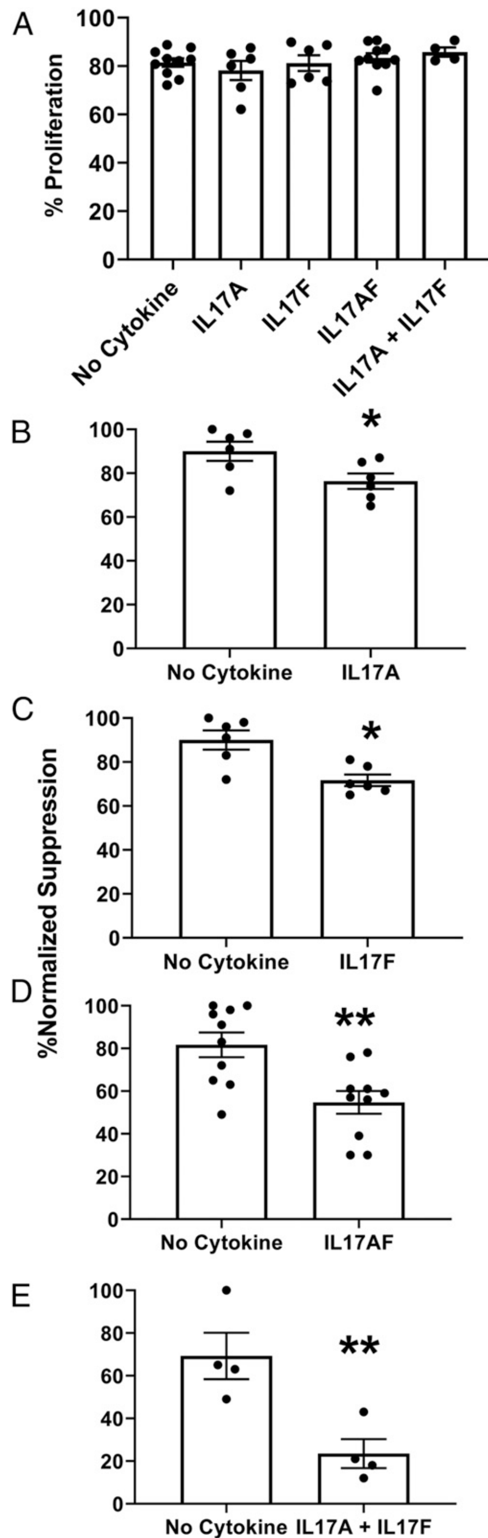

Fig. 3. Exposure of bulk (non-Th17) CD4+ T cells to exogenous IL-17A, IL-17F, or IL-17AF results in acquisition of resistance to immune suppression. Bulk ex vivo CD4+CD25- T cells were activated in the presence of indicated IL-17 cytokines (10 ng/mL each) for 7 d, followed by washing, CFSE staining, and suppression assays using autologous APC, CD8 T cells, and fixed αCD3. (*A*) represents %proliferation (mean ± SEM) of these cells in the absence of CD8+ T cells (1:0 condition). *B*–*E* represent mean %suppression ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

Therefore, we next investigated the potential mechanism of this functional change. For this, we activated ex vivo-purified bulk CD4+CD25- T cells for 48 h in the presence or absence of IL-17A, IL-17F, or IL-17AF and performed transcriptome analysis using RNA sequencing (RNA-seq). To confirm our flow cytometric observations that T cells express known IL-17 receptors, we specifically looked at *IL17RA* and *IL17RC* message within the RNA-seq data. We found expression of both receptors in these cells, with *IL17RA* expression significantly greater than *IL17RC* (*SI Appendix*, Fig. S6*A*), corroborating with the flow cytometry data shown in *SI Appendix*, Fig. S5. We did not see significant changes in the expression of either receptor following exposure to any of the IL-17 cytokines (*SI Appendix*, Fig. S6 *B* and *C*).

Using the differential expression comparisons between each of the experimental conditions, we performed Ingenuity Pathway Analysis (IPA). Across all three conditions, we saw a number of pathways significantly increased (Fig. 4*A*) and far fewer pathways decreased (*SI Appendix*, Fig. S7*A*). To address the common factors that may be responsible for shared pathways, we next quantified the appearance of signaling molecules across all of the significantly different pathways for each condition (Fig. 4*B*). Although some variations in ranking of the molecules existed between conditions, we found IL-1B and IL-6 pathways to be most likely drivers of the genetic alterations seen in the pathway analysis. IPA also performs estimated enrichments for upstream regulators of distinct genetic programs. We examined the upstream regulators and found decreased (*SI Appendix*, Fig. S7*B*) and increased (*SI Appendix*, Fig. S7*C*) enrichment across the IL-17A, IL-17F, or IL-17AF conditions.

These results suggested that IL-17 could act directly on CD4+ T cells to induce changes in multiple pathways. Since changes in IL-1B and IL-6 pathways were most numerous, we decided to directly test whether either of these cytokines was involved in the suppressive resistance of these cells. Prior studies have implicated the IL-6/STAT3 pathway in effector CD4 resistance (16). Therefore, in addition to IL-1β and IL-6 blockade, we also included the STAT3 inhibitor, STATTIC V, during suppression assays. Ex vivo-purified bulk CD4+ CD25− were subjected to suppression assays either in media or in the presence of IL-17A + IL-17F. No APCs were used in these assays, with anti-CD3/anti-CD28-coated beads providing the stimulus, to ascertain that we were assessing T cell-intrinsic phenomena. These assays were conducted in the presence (or absence) of anti-IL-1β, anti-IL-6 (singly or in combination) or STATTIC V. As seen in Fig. 4 *C* and *D*, in the absence of inhibitors, the addition of IL17A+IL17F resulted in greatly enhanced resistance to suppression compared to control (similar to that in Fig. 3*E*). The presence of inhibitors did not alter the suppressibility of control conditions (Fig. 4*C*). However, blockade of IL-1β, IL-6, or STAT3 resulted in significant reversal of CD4 resistance (Fig. 4*D*), indicating that these pathways play an important role in IL-17–mediated effector resistance. Interestingly, each of the inhibitors resulted in similar reversal, suggesting that these pathways may be part of a connected cascade of events or feedback loops.

In this study, we have uncovered three fundamental concepts: 1) Different lineages of CD4+ T cells have differential susceptibility to immune suppression, with Th17 cells showing high resistance; 2) the signature Th17 cytokines, IL-17A, IL-17F, and IL-17A/F, play an important role in mediating this immune resistance by acting directly on CD4+ T cells in an autocrine/paracrine manner; and 3) IL-17 mediates CD4 resistance through the IL-1β and/or IL-6/STAT3 pathways.

Th17 cells and their cytokines are of immense interest in various settings of disease and health. For example, secukinumab, an agent targeting IL-17A, is a Food and Drug Administration-approved therapy for psoriasis, psoriatic arthritis, ankylosing spondylitis, but has not shown promising results in other autoimmune disease

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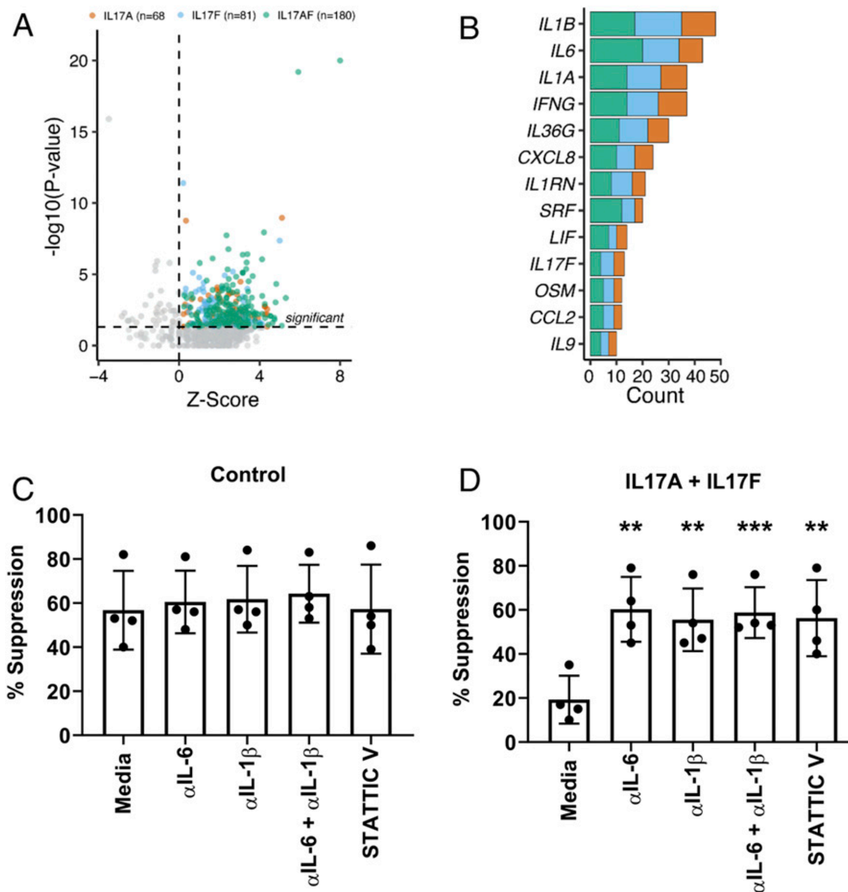

Fig. 4. (*A*) IL-17 exposure induces IL-6– and IL-1β–related pathways, which, in turn, mediate effector CD4 T cell resistance to suppression. Canonical pathway enrichment using IPA for IL-17A–, IL-17F–, and IL-17AF–treated CD4+CD25- cells, compared to media alone, using RNA-seq data derived from each condition ($n = 3$). Highlighted pathways had a $P < 0.05$ and a Z-Score > 0. (*B*) The instances of cytokine or transcriptional factor molecules appearing in the significantly enriched canonical pathways from *A* by IL-17A, IL-17F, and IL-17AF conditions. Ex vivo-purified CD4+CD25- T cells were cultured for 7 d in either media alone (*C*, control) or in the presence of IL17A+IL17F (*D*). These cells were then placed in suppression assays following either the addition of anti-IL6, anti-IL1β (singly or combination), or pretreatment with the STAT3 inhibitor V STATTIC. Column bars represent %suppression ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

settings. While the design of such agents has been based on the known functions of IL-17 cytokines in mediating immune pathology, the mechanistic dissection of their effects (and potential failures) should take into account the other effects of these cytokines, including their potential role in immune resistance. Of particular note is our observation that IL-17A, IL-17F, and IL-17A/F, regardless of their source, may induce resistance in neighboring non-Th17 CD4+ T cells. It is tempting to speculate that such resistance may be important in mediating immune responses to infection and cancer, while the same mechanism may result in greater pathology when employed by autoaggressive CD4+ T cells that have differentiated in a pathogenic cytokine milieu.

In our studies, we generated resistant Th17 cells from naïve CD4+ T cells by using a combination of TGF-β1, IL-1β, and IL-6 along with blockade of IL-4 and IFNγ, with anti-CD3/anti-CD28 stimulation. Other combinations have also been used in prior reports, particularly inclusion of IL-23 to generate pathogenic Th17 cells (23, 24, 30, 31) or omission of CD28 costimulation to enhance the percentage of IL-17–producing cells in these cultures (32, 33). In future studies, it will be important to understand the roles of these individual cytokines and stimuli in not only promoting IL-17A and IL-17F production but also in imparting suppressive resistance in the CD4+ T cells. This will allow dissection of individual pathways that generate these complex characteristics. Similarly, the effect of IL-17 cytokines on T cells within an inflammatory disease context may induce an even broader interplay of cellular pathways. In our study, we have demonstrated that these cytokines can act directly on ex vivo-derived CD4+ T cells, which is a mixture of naïve and memory/effector T cells, comprising predominantly non-Th17 cells. This provides a framework for future studies where the effects of these cytokines on predifferentiated T helper cell types can be determined. In that context, lineages of T helper cells other than Th1, Th2, and Th17, such as granulocyte-macrophage colony-stimulating factor-producing Th cells, which play important roles in disease pathogenesis (34–36), can also be evaluated as potential targets of these cytokines. Similarly, it will also be important to dissect the effects of these combinations of inflammatory conditions on the function of CD8+ T cells themselves. While the bulk CD8+ T cells added to these cultures do not have an appreciable fraction of IL-17A–producing cells (0.43% ± 0.13% of CD8 + T cells were IL-17A+), it is possible that the various Th cells modulate the CD8+ T cells in different ways. This is an intense focus on ongoing and future studies in our laboratory.

IL-17A, IL-17F, and the IL-17AF heterodimer bind to IL-17RC with comparable affinities, whereas they bind to IL-17RA with different affinities. Some studies have shown that IL-17A activity is inhibited by soluble IL-17RA, IL-17F is inhibited by soluble IL-17RC, and a combination of soluble IL-17RA/IL-17RC receptors is required for inhibition of the heterodimer activity. Thus, all three cytokines seem to act through the same receptor complexes, but the distinct affinities of the receptor components can differentially affect the activity of these cytokines

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(37). In our studies, we saw that blockade of IL-17A, IL-17F, or IL-17AF reversed the resistance of CD4+ T cells. Similarly, blockade of IL-17RA or IL-17RC also reversed the resistance, with the greatest reversal seen when both receptors were blocked. This suggests that induction of resistance is either a shared function of all three cytokines or is a unique function of the dimer that requires binding to both IL-17RA and IL-17RC. Interestingly, our data indicate that this binding has to take place directly on the CD4+ T cell to induce resistance, since blockade of the receptors on either APC or CD8+ T cells did not reverse the resistant phenotype. This indicates an interesting autocrine/paracrine pathway that would be an important pursuit in future studies.

Of particular interest is the involvement of IL-6R/IL-6/phospho-STAT3 pathways in the induction of suppressive resistance in CD4+ T cells in the context of multiple sclerosis and other autoimmune diseases (16, 38, 39). Interestingly, IL-17A is implicated in further inducing IL-6 secretion through its action on both IL-17RA and IL-17RC (29). Our observation that T cells may also be the source of IL-1β and IL-6, which can act directly on T cells to effect resistance may indicate a feedback loop in generating CD4 resistance that may be amenable to therapeutic intervention by using a combination of cytokine blockade.

Methods

Cell Preparation and Bead Sorting. PBMCs from healthy subjects were isolated from deidentified leukocyte reduction system (LRS) cones containing leukocyte-rich whole blood from platelet donors at the University of Iowa, DeGowin Blood Center. PBMC isolation was performed with BD Vacutainer CPT tubes (BD, 362753) density gradient centrifugation. CD8 T cells were positively selected from freshly prepared PBMCs with Manual LS Column MACS sorting with Miltenyi Biotech MACS Bead sorting microbeads (130-045-201) according to manufacturer specifications. Untouched Naïve CD4+ T cells were negatively selected from the remaining PBMCs with either Naïve CD4+ T cell Isolation Kit (130-094-131) or CD4+ T cell Isolation Kit (130-096-533) followed by a CD45RO depletion. Sort purities were routinely above 95% by flow cytometric analysis (*SI Appendix*, Fig. S1*A*). Sorted CD8 T cells, CD4+ T cells, and CD4/CD8-depleted PBMC (used as APC) were frozen in dimethyl sulfoxide-containing media on the day of sorting for future use.

Th Subset Differentiation. Naïve CD4+ T cells were thawed in RPMI 1640 (Corning 10–040-CV) with DNase at 10 KU/mL (Sigma D4513-1vl) and then resuspended at 1 × 10e6 cells per milliliter in X-VIVO 15 serum-free media (Lonza, 04-418Q), followed by stimulation in various differentiation conditions (Media Alone/Th0, Th1, Th2, Th17), as shown in *SI Appendix*, Fig. S1*B*. Conditions were based on previous publications (22–24) and included: 1) Media Alone/Th0: no cytokines/antibodies added; 2) Th1: anti-IL-4 7 μg/mL BD554481, IL-2 10 ng/mL BD554603, IL-12 10 ng/mL BD554613; 3) Th2: anti-IFNγ 7 μg/mL BD554698, IL-2 10 ng/mL, IL-4 10 ng/mL BD554605; 4) Th17: anti-IL4 7 μg/mL, anti-IFNγ 7 μg/mL, TGFβ1 10 ng/mL eBioscience 14–8348-62, IL-1β 10 ng/mL BD554602, IL-6 50 ng/mL BD550071. Cultures were activated with 1 μg/mL each of fixed anti-CD3 (eBioscience, 16-0037-85) and anti-CD28 (eBioscience, 16-0289-85), as described previously (40) and incubated for 7 d at 37 °C. Supernatants were aliquoted at day 7 for enzyme-linked immunosorbent assays (ELISAs), and cells were washed twice with phosphate-buffered saline (PBS) for suppression assay cultures. In some experiments, an aliquot of cultured cells was used for intracellular cytokine staining to assess their state of differentiation.

ELISA. ELISA was performed on supernatants per manufacturer protocol (eBioscience Human Platinum ELISA Kits for IFNγ [BMS228], IL-5 [BMS278], IL-10 [BMS215/2], IL-13 [BMS231/3], IL-17A [BMS2017], IL-17F [BMS2037-2], and IL-17AF [BMS2082]). ELISA data were acquired on a BioTek Synergy H1 Hybrid Reader. Gen5 v2.09 was used for software analysis.

Intracellular Flow Cytometric Cytokine Assays. For surface and intracellular staining on day 7 of in vitro differentiation, cells were washed in PBS and then cultured in media with 2 μL of Leukocyte Activation Mixture with Golgi Plug (BD, 550583) for 5 h, followed by washing with 0.1% (wt/vol) sodium azide/PBS and surface staining with anti-CD3 APC (BioLegend, 300458) and anti-CD4 BV786 (BD, 563877). Cells were fixed overnight at 4 °C followed by permeabilization using eBioscience fixation/permeabilization kit. Intracellular staining was performed using anti-IFNγ AlexaFluor700 (BD, 557995). All cells were resuspended in staining buffer (0.1% [wt/vol] sodium azide/PBS) for FACS analysis. Flow cytometric data were acquired on a 4-Laser, 17-color LSRII using BD FACSDiva Software v6.1.3 (Firmware v1.9). FlowJo version 9.1 was used for analysis.

Flow Cytometric Suppression Assays. CD4+ T cells from the 7-d differentiation were placed in flow cytometric suppression assays, as described previously (12, 13). Briefly, responder CD4+ T cells were stained with CFSE, followed by culture with irradiated APCs and 1 μg/mL fixed anti-CD3 (eBioscience, 16-0037-85) in the presence or absence of ex vivo sorted autologous bulk CD8+ T cells. On day 7 of culture, cells were stained for anti-CD4 PE-Cy7 (BD, 557852), anti-CD3 AlexaFlour700 (BD, 557943), anti-CD8 Pacific Blue (Biolegend, 344718), and flow cytometrically assessed for CD4 proliferating fraction (CFSE dilution). % proliferation and %suppression were calculated as described previously (12). As indicated in some of the assays, the following neutralizing antibodies were added at 7 μg/mL: anti-IL-17A (eBioscience, 16-7178-85), anti-IL-17F (eBioscience, 16-7169-85), anti-IL-17AF (R&D Systems, AF317-NA), anti-IL-21 (LSBio, LS-C104584), anti-IL-22 (eBioscience, 16-7222-85). In some experiments, recombinant human cytokines IL17A (eBioscience, 34-8179-82), IL17F (R&D Systems, 1335-IL-025/CF)- and IL17AF (R&D Systems, 5194-IL-025/CF) were used at 10 ng/mL each. In case of combined cytokines, each was at 10 ng/mL. For experiments involving IL-17 receptor blockade, cultured CD4+ T cells, thawed CD8 T cells, and APCs were first incubated with 7 μg/mL of anti-IL17RA (eBioscience, 16-7917-85) and/or anti-IL17RC (Abcam, ab69673) for 90 min at 25 °C. Cells were then washed and used in suppression assays.

IL-1β, IL-6/STAT3 Pathway Blockade. CD4+ CD25- T cells were magnetically sorted and stimulated with αCD3 and αCD28 as above, either in media alone or with a combination of recombinant carrier free human cytokines IL-17A (BioLegend, 570506) and IL-17F (R&D Systems, 1335-IL-025/CF) at 10 ng/mL each for 7 d. Cells were then CFSE stained and incubated with either the STAT3 inhibitor, STATTIC V (Santa Cruz Biotechnology, SC-202818), at 200 ng/mL for 1 h followed by a PBS wash, or with 7 μg/mL of neutralizing anti-IL6 (BD Pharmingen, 554541), anti-IL1β (InvivoGen, magb-hil1b-3), or the combination of anti-IL6 + anti-IL1β. These cells were placed in suppression assays with bulk CD8+ suppressor T cells, as described above.

RNA-Seq/Transcriptome Analysis. Ex vivo-purified bulk CD4 + CD25- T cells were activated in vitro for 48 h in the presence of media alone (controls), or 10 ng/mL IL-17A, IL-17F, or IL-17AF. Samples were submitted to the University of Chicago Genomics facility for RNA extraction, quality assessment, and sequencing. Single-end 50-bp sequencing was performed on the Illumina HiSeq 2000. Basecalls were converted into FASTQs using the Illumina bcl2fastq and alignment was performed using kallisto (41) with the GRCh38 human genome build. Pseudoalignments were processed using sleuth (v0.30) R package (41). Differential gene expression analysis was performed in the sleuth R package with the Wald test. Differential genes were defined as $\log_2$-fold change >1 or <−1 and false discovery rate < 0.05. The significant genes were used for IPA (Qiagen) using the same cut points for significance as inputs.

Statistics. GraphPad Prism v7.03 was used for statistical analyses (mostly paired *t* tests). $P < 0.05$ was considered significant.

Study Approval. All experiments were performed on PBMCs obtained from deidentified LRS cones from healthy platelet donors at the University of Iowa DeGowin Blood Center, as approved by the University of Iowa IRB.

Data Availability. All data are included in this article, with the exception of raw RNA-seq data, which have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession no. GSE150805.

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