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Self-Sustained Resistance to Suppression of CD8+ Teff Cells at the Site of Autoimmune Inflammation Can Be Reversed by Tumor Necrosis Factor and Interferon-γ Blockade

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Objective. **Resistance of Teff cells to Treg cell–mediated suppression contributes to the breakdown of peripheral tolerance in the inflamed joints of patients with juvenile idiopathic arthritis (JIA). However, unanswered questions are whether this resistant phenotype is self-sustained and whether CD8+ and CD4+ Teff cells share the same mechanism of resistance to suppression. We undertook this study to investigate intrinsic resistance of CD8+ Teff cells to suppression and to determine how this can be targeted therapeutically.**

Methods. **CD8+ or CD4+ Teff cells were cultured with or without antigen-presenting cells (APCs) in Treg cell–dependent and –independent suppression assays. Synovial fluid (SF)–derived Teff cells were crosscultured with peripheral blood (PB) Treg cells from JIA patients or healthy controls. Tumor necrosis factor (TNF) or interferon-γ (IFNγ) blocking agents were used to restore Teff cell responsiveness to suppression.**

Results. **Suppression of cell proliferation and cytokine production in CD8+ Teff cells from the SF of JIA patients was severely impaired compared to that in CD8+ Teff cells from the PB of JIA patients, regardless of the presence of APCs and CD4+ Teff cells. Similar to CD4+ Teff cells, impaired suppression of CD8+ Teff cells was shown to be an intrinsic feature of this cell population. While TNF blockade restored both CD8+ and CD4+ Teff cell susceptibility to suppression, autocrine release of IFNγ selectively sustained CD8+ Teff cell resistance, which could be relieved by IFNγ blockade.**

Conclusion. **Unlike CD4+ Teff cells, resistance of CD8+ Teff cells to suppression at the site of autoimmune inflammation is maintained by autocrine release of IFNγ, and blockade of IFNγ restores CD8+ Teff cell responsiveness to suppression. These findings indicate a potential therapeutic value of blocking IFNγ to restore immune regulation in JIA.**

Autoimmune diseases such as rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) are characterized by an aberrant immune response toward self antigens, leading to severe tissue damage (1,2). Both diseases feature infiltration of the synovial tissue by immune cells, proliferation of synoviocytes, and accumulation of synovial fluid (SF) in the joint, causing swelling and limitation of motion (2). Among other cell populations, the target organ is typically massively infiltrated by T cells, which display an activated phenotype and maintain a proinflammatory environment (2). Under conditions of health, latent autoreactive Teff cells can be successfully controlled by mechanisms of peripheral tolerance, such as Treg cell–mediated suppression (3). However, in autoimmune diseases, the breakdown of peripheral tolerance (4,5) leads to insufficient control of Teff cells and Teff cell–induced pathology.

Our group has recently shown that Teff cells from the SF of JIA patients, but not those from peripheral blood (PB), are refractory to Treg cell–mediated suppression due to Akt hyperphosphorylation (6). In addition,

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we showed that responsiveness to suppression could be successfully restored by anti–tumor necrosis factor (anti-TNF) in vitro (7). Since antigen-presenting cells (APCs) are a likely source of TNF, we wondered whether the resistant phenotype of CD4+ and CD8+ Teff cells is self-sustained and can be maintained by the absence of APCs in culture. Haufe et al recently demonstrated that purified CD4+ Teff cells from the SF of patients with RA were intrinsically resistant to suppression, suggesting that the resistant phenotype of CD4+ Teff cells is maintained in the absence of proinflammatory cytokines produced by APCs (8). However, it remains to be explored what mechanism underlies this self-sustained resistance of CD4+ Teff cells to suppression.

To date, investigation of CD4+ T cells has been of great interest to the scientific community (8,9). However, CD8+ Teff cells are relevant in arthritis, are known to be enriched in the SF of patients with RA (10,11), and contribute to proinflammatory cytokine production (11). SF CD8+ T cells are also resistant to suppression, but it remains to be explored whether this resistance to suppression is cell intrinsic and independent of the presence of CD4+ Teff cells and APCs, and whether CD8+ Teff cell resistance can be self-sustained. Defining the mediators of Teff cell self-sustained resistance to suppression at the site of autoimmune inflammation is of particular relevance in the context of drug-induced Treg cell expansion or Treg cell–based therapies, for which coadministration of a specific drug targeting Teff cell resistance will be a prerequisite for successful treatment.

In the present study, we investigated whether, in a manner similar to that of CD4+ Teff cells, CD8+ Teff cell resistance to suppression at the site of autoimmune inflammation in JIA is intrinsic and what are the soluble mediators of CD8+ and CD4+ Teff cell self-sustaining resistance to suppression. We found that CD8+ Teff cells residing in the SF of JIA patients are intrinsically resistant to suppression, independently of the presence of CD4+ T cells. TNF and interferon-γ (IFNγ) redundantly sustain this resistance of CD8+ Teff cells to suppression, whereas CD4+ Teff cells are affected by TNF only. This study reveals a difference between CD4+ and CD8+ Teff cells in the cytokine requirements maintaining their resistant state and demonstrates a therapeutic potential for IFNγ blockade in restoring CD8+ Teff cell responsiveness to suppression.

PATIENTS AND METHODS

Patient population. Patients with JIA were enrolled by the pediatric rheumatology department at University Medical Center Utrecht (Utrecht, The Netherlands) after providing written informed consent either directly or from parents/guardians if they were <12 years old. Patients with oligoarticular JIA (n = 12) and patients with polyarticular JIA (n = 6) according to the revised criteria for JIA (12) were included in this study. The study was conducted in accordance with local ethics committee approval (protocol Pharmachild) and the Declaration of Helsinki. The mean age of the patient population was 13.8 years (range 8–17 years) and the mean disease duration at the time of inclusion was 5.5 years (range 1–13 years). SF was obtained from 10 patients with active disease undergoing therapeutic joint aspiration. Paired blood and joint aspirate samples were collected from 4 patients. PB only was collected from 4 patients. Patients were either untreated (n = 4) or treated with nonsteroidal antiinflammatory drugs (NSAIDs) alone (n = 3), NSAIDs plus methotrexate (MTX) (n = 5), MTX alone (n = 4), or MTX plus <0.3 mg/kg/day oral prednisolone (n = 2) at the time of inclusion. Paired samples were collected from 1 untreated patient, 1 patient treated with NSAIDs alone, and 2 patients treated with MTX alone. Healthy control buffy coats (n = 10) from adult volunteers were obtained from Sanquin Blood Bank (Amsterdam, The Netherlands).

Cell isolation. Synovial fluid mononuclear cells (SFMCs) were incubated with hyaluronidase (Sigma-Aldrich) for 30 minutes at 37°C. SFMCs and peripheral blood mononuclear cells were isolated using Ficoll Isopaque density-gradient centrifugation (GE Healthcare Biosciences) and frozen in fetal calf serum (FCS) (Invitrogen) containing 10% DMSO (Sigma-Aldrich) until further experimentation.

Cell culture and reagents. CD8+ and CD4+CD25−CD127^{high} T cells (25,000 cells/100 μl) were cultured in RPMI 1640 supplemented with 2 m*M* L-glutamine, 100 units/ml penicillin/streptomycin, and 10% human AB serum (all from Invitrogen) at 37°C and an atmosphere of 5% CO_2 in round-bottomed 96-well plates (Nunc). Cells were stimulated with anti-CD2/CD3/CD28–coated beads (Treg Suppression Inspector; Miltenyi Biotec) in some experiments or with CD3− cells as APCs. Each batch of Treg Suppression Inspector beads was titrated before performing experiments and adjusted at a cell-to-beads ratio to obtain a minimum 20% cell proliferation. Under some conditions, anti-human TNF (etanercept) and anti-human IFNγ (eBioscience) were added at 1 μg/ml.

Suppression assay. CD3− cells (APCs), CD4+CD25+CD127^{low} Treg cells, and CD8+ and CD4+CD25−CD127^{high} T cells in the PB and SF of JIA patients and the PB of healthy controls were sorted by flow cytometry on a FACSAria III (BD Biosciences). CD8+ and CD4+CD25−CD127^{high} T cells were used as responder cells and are called CD8+ and CD4+ Teff cells, respectively, throughout. Teff cells were labeled with CellTrace Violet fluorescent dye (Invitrogen) to measure proliferation by dye dilution. Treg cells were cocultured with Teff cells at a 1:2 (Treg cell:Teff cell) ratio and stimulated with either Treg Suppression Inspector beads or CD3− cells at a 1:1 (APC:Teff cell) ratio. In crossover assays, autologous Treg cells from the PB of JIA patients or allogeneic Treg cells from healthy controls were cocultured with Teff cells from the SF of JIA patients. Treg cell–independent suppression of Teff cells from the SF of JIA patients and the PB of healthy controls was performed by replacing Treg cells with 50 ng/ml transforming growth factor β (TGFβ) (PeproTech). On day 4, proliferation of Teff cells was analyzed by flow cytometry and supernatant was collected to measure cytokine production.

Cytokine measurement. Supernatants were collected from suppression assays after 96-hour cultures, and levels of IFNγ, TNF, interleukin-6 (IL-6), IL-17, and IL-10 were measured using Luminex technology as previously described (6).

Flow cytometry. To detect intracellular cytokine production, cells were stimulated for 4 hours with phorbol myristate acetate (PMA) (20 ng/ml; MP Biomedicals) and ionomycin (1 μg/ml; Calbiochem), with GolgiStop (1:1,500; BD Biosciences) added for the last 3.5 hours of culture. Before staining, cells were washed twice in fluorescence-activated cell sorting (FACS) buffer (phosphate buffered saline containing 2% FCS [Invitrogen] and 0.1% sodium azide [Sigma-Aldrich]) and subsequently incubated with surface antibodies (BV510-conjugated anti-human CD3, Alexa Fluor 488–conjugated anti-CD4, allophycocyanin–Cy7–conjugated anti-CD8). After surface staining, cells were washed twice in FACS buffer and fixed, permeabilized, and intracellularly stained using fluorescein isothiocyanate–conjugated anti-human TNF and phycoerythrin–Cy7–conjugated anti-IFNγ. To determine Treg cell purity after sorting, sorted Treg cells were washed twice with FACS buffer, fixed, permeabilized, and intracellularly stained using an anti-human FoxP3 staining set (eBioscience) according to the manufacturer's instructions.

Statistical analysis. For statistical comparison between 2 unpaired groups, the Mann-Whitney U test was used. We used the Wilcoxon signed rank test to analyze paired samples. *P* values less than 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism software.

RESULTS

SF CD8+ Teff cells are intrinsically resistant to suppression regardless of the presence of APCs and CD4+ T cells. We (6) and others (8,13) have previously shown that Teff cells from the site of chronic autoimmune inflammation in JIA patients (i.e., the SF) are resistant to Treg cell–mediated suppression of proliferation and cytokine production in the presence of APCs. However, in those studies, the main focus was on CD4+ Teff cells, and whether CD8+ Teff cells are resistant to suppression independently of the presence of CD4+ Teff cells and APCs has not been investigated. To investigate this, we cultured purified CD8+ Teff cells from the SF of JIA patients and compared suppression by Treg cells when using either APCs from the same site (i.e., the SF) or anti-CD2/CD3/CD28–coated beads as stimulators. Suppression of CD4+ Teff cells was used as a reference. SF Treg cells were sorted (see Supplementary Figure 1A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39418/abstract) and tested for purity by staining for FoxP3, the expression of which was confirmed to be higher than that on CD4+ Teff cells (see Supplementary Figure 1B).

Purified CD8+ Teff cells from the SF of JIA patients showed enhanced proliferation compared to cells from the PB of JIA patients when cultured with APCs, but not when stimulated with beads (Figure 1A). No differences in cell proliferation were evident in the CD4+ Teff cell compartment (Figure 1A). Suppression of proliferation of CD8+ Teff cells from SF was impaired compared to suppression of proliferation of cells from PB, both in the presence and absence of APCs (Figure 1B). However, CD8+ Teff cell proliferation did not correlate with suppression levels (data not shown). Consistent with the findings of Haufe et al, suppression of proliferation of CD4+ Teff cells from SF was also impaired both in the presence and absence of APCs (Figure 1B). Representative histograms showing CD8+ and CD4+ Teff cell proliferation and suppression are shown in Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39418/abstract.

To elucidate whether the impaired Treg cell–mediated suppression observed in the APC-independent assay applies not only to Teff cell proliferation but also to cytokine release, we tested cytokine levels in the supernatant of suppression assays with sorted CD8+ or CD4+ Teff cells stimulated with anti-CD2/CD3/CD28–coated beads (Figure 1C). Consistent with the proliferation data, cytokine production was suppressed when Treg cells from the PB of JIA patients were cocultured with CD8+ Teff cells from the same site (Figure 1C), but no suppression of cytokine production by CD8+ Teff cells from SF was observed (Figure 1C). Although not statistically significant, the same trend was observed when CD4+ Teff cells were used as responders (Figure 1C). Levels of IL-6 and IL-17 were also measured, but they were below the detection limit under most of the study conditions. Overall, these data show that, similar to CD4+ Teff cells, CD8+ Teff cells from sites of autoimmune inflammation in JIA patients are intrinsically resistant to Treg cell–mediated suppression regardless of the presence of APCs and CD4+ T cells.

CD8+ Teff cells from SF of JIA patients are intrinsically resistant to suppression even in the presence of functional Treg cells or an immunosuppressive cytokine. To further confirm that the resistance to suppression of SF CD8+ Teff cells is purely Teff cell dependent and not the result of interplay with SF Treg cells, we investigated suppression of SF CD8+ Teff cells by different sources of Treg cells. Given the ability of Treg cells to suppress equally whether derived from the PB of JIA patients or from the PB of healthy adult controls (6), we first performed an autologous crossover suppression assay using Treg cells from the PB of JIA patients cocultured with paired sorted CD8+ or CD4+ Teff cells from the SF of JIA patients and stimulated with beads. Consistent with previously reported data (6), PB-derived Treg cells from JIA patients were perfectly capable of suppressing PB-derived CD8+ Teff cells. However, even in the presence

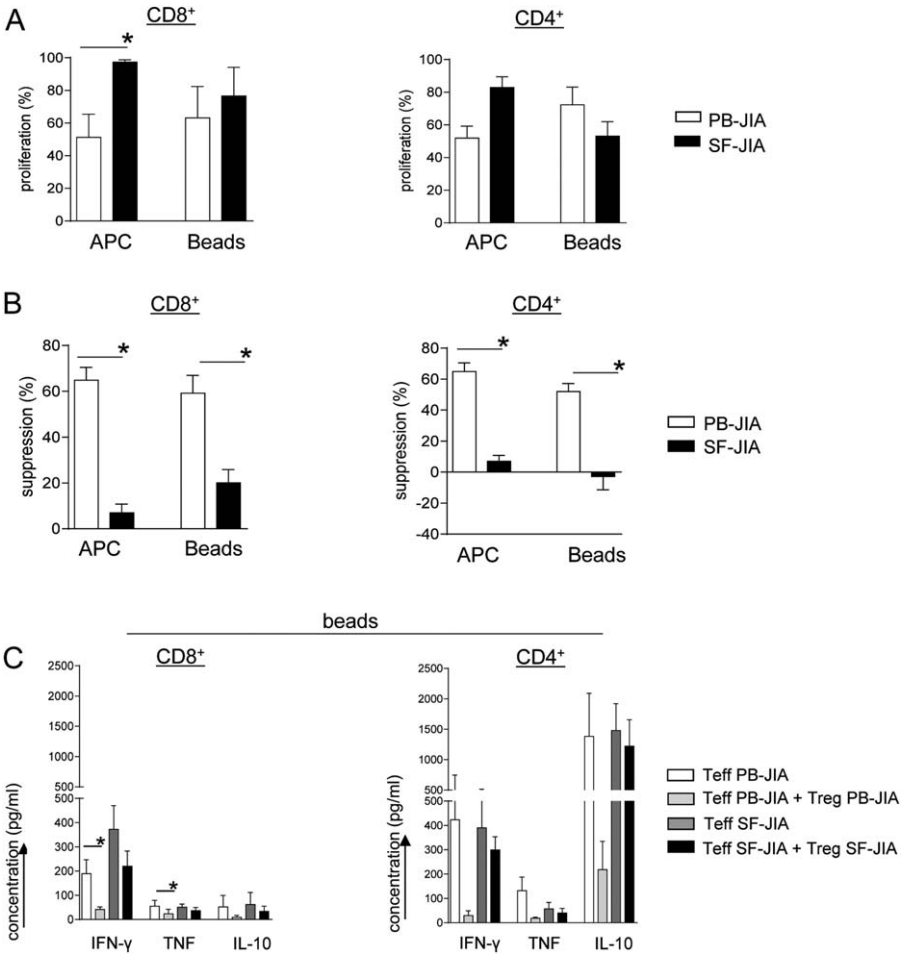

Figure 1. Synovial fluid (SF)–derived CD8+ Teff cells display intrinsic resistance to suppression of cell proliferation and cytokine production independent of CD4+ Teff cells or the presence of antigen-presenting cells (APCs). **A,** Proliferation of CellTrace Violet–labeled CD8+ and CD4+ Teff cells from the peripheral blood (PB) and SF of patients with juvenile idiopathic arthritis (JIA) was measured in the presence of APCs (CD3− cells) from the same site (i.e., from PB or SF, respectively) or anti-CD2/CD3/CD28–coated beads as stimulators. **B,** CellTrace Violet–labeled CD8+ Teff cells from the PB and SF of JIA patients stimulated with APCs (6 PB samples and 3 SF samples) from the same site (i.e., from PB or SF, respectively) or with anti-CD2/CD3/CD28–coated beads (5 PB samples and 6 SF samples) were cocultured in the presence or absence of Treg cells from the same site (i.e., from PB or SF, respectively). Treg cell–mediated suppression of CD8+ Teff cell proliferation after 96-hour coculture is shown. Treg cell–mediated suppression of CD4+ Teff cell proliferation was tested in parallel. **C,** Cytokine levels in the supernatant after 96-hour coculture using anti-CD2/CD3/CD28–coated beads in the presence of CD8+ and CD4+ Teff cells were measured by Luminex assay. Values are the mean ± SEM. $* = P < 0.05$. IFNγ = interferon-γ; TNF = tumor necrosis factor; IL-10 = interleukin-10.

of these PB-derived Treg cells, CD8+ Teff cells from SF were resistant to suppression (Figure 2A). CD4+ Teff cells behaved in a similar manner (Figure 2A). Representative histograms of SF Teff cell proliferation and suppression are shown in Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39418/abstract.

Although autologous Treg cells from the PB of JIA patients were able to suppress PB-derived Teff cells, we cannot exclude the possibility of a reduced functionality of these cells that remained undetected due to limited sensitivity of the assay. Therefore, we also performed an allogeneic suppression assay using functional Treg cells from the PB of healthy adult controls. In 6 of 8 SF samples, CD8+ Teff cell proliferation was not suppressed upon coculture with functional Treg cells from healthy adult controls (Figure 2B). There was no evident correlation between the restoration of suppression occurring in the 2 SF samples and the clinical features of the patients. Although donor-to-donor variability was evident in the presence of SF-derived CD4+ Teff cells as well (Figure 2B), suppression by coculture with Treg cells from healthy controls was significantly reduced compared to the reference condition, indicating

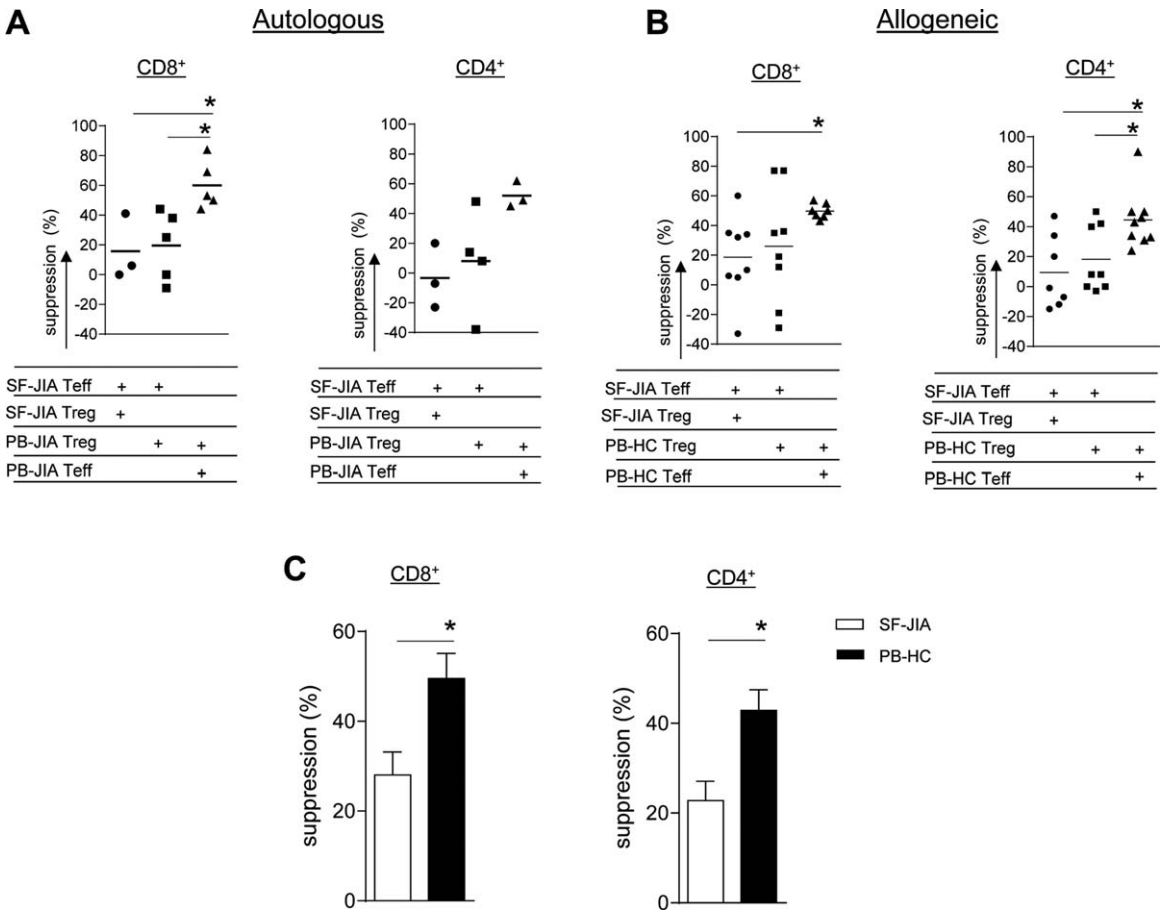

Figure 2. Intrinsic resistance of synovial fluid (SF)–derived CD8+ Teff cells to suppression even in the presence of functional Treg cells or an immunosuppressive cytokine. **A,** Antigen-presenting cell–independent, Treg cell–mediated suppression of CD8+ Teff cells from the SF of patients with juvenile idiopathic arthritis (JIA) was measured in the presence of autologous Treg cells collected from the peripheral blood (PB) of JIA patients (n = 5 samples). Reference values (third column in each graph) represent the suppression of Teff cells from the PB of JIA patients by autologous Treg cells (n = 5 samples). CD4+ Teff cell suppression was tested in parallel. **B,** Treg cell–mediated suppression of CD8+ Teff cells from the SF of JIA patients (n = 8 samples) was measured in the presence of allogeneic Treg cells collected from the PB of healthy adult controls (HC) (n = 7 samples). CD4+ Teff cell suppression was measured in parallel. Reference values (third column in each graph) represent the suppression of Teff cells from the PB of healthy controls by allogenic Treg cells. **C,** Treg cell–independent suppression of proliferation of CD8+ Teff cells from the SF of JIA patients (n = 5 samples) and the PB of healthy controls (n = 8 samples) was measured by coincubation with transforming growth factor β (TGFβ). TGFβ-mediated suppression of proliferation of CD4+ Teff cells was tested in parallel. In **A** and **B**, symbols represent individual samples; horizontal lines indicate the mean. In **C**, values are the mean ± SEM. $* = P < 0.05$.

that resistance of CD4+ and CD8+ Teff cells to suppression is independent of the Treg cell source. This was also confirmed by the absence of suppression of cytokine levels in the supernatant of the autologous (see Supplementary Figure 4A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39418/abstract) and allogeneic (see Supplementary Figure 4B) assays.

In addition, in a Treg cell–independent suppression assay in which Treg cells were replaced with TGFβ, a well-known mediator of suppression (14), we found impaired suppression of SF-derived CD8+ Teff cells from JIA patients compared to PB-derived CD8+ Teff cells from healthy adult controls (Figure 2C). The same was evident for CD4+ Teff cells (Figure 2C). Both CD8+ and CD4+ Teff cells from the SF of JIA patients were also found to be enriched in memory cells and showed a hyperactivated state, as indicated by increased expression of CD45RO, CD25, and CD69 as compared to CD8+ and CD4+ Teff cells from the PB of JIA patients (see Supplementary Figure 5, available on the *Arthritis & Rheumatology* web site at http://online-

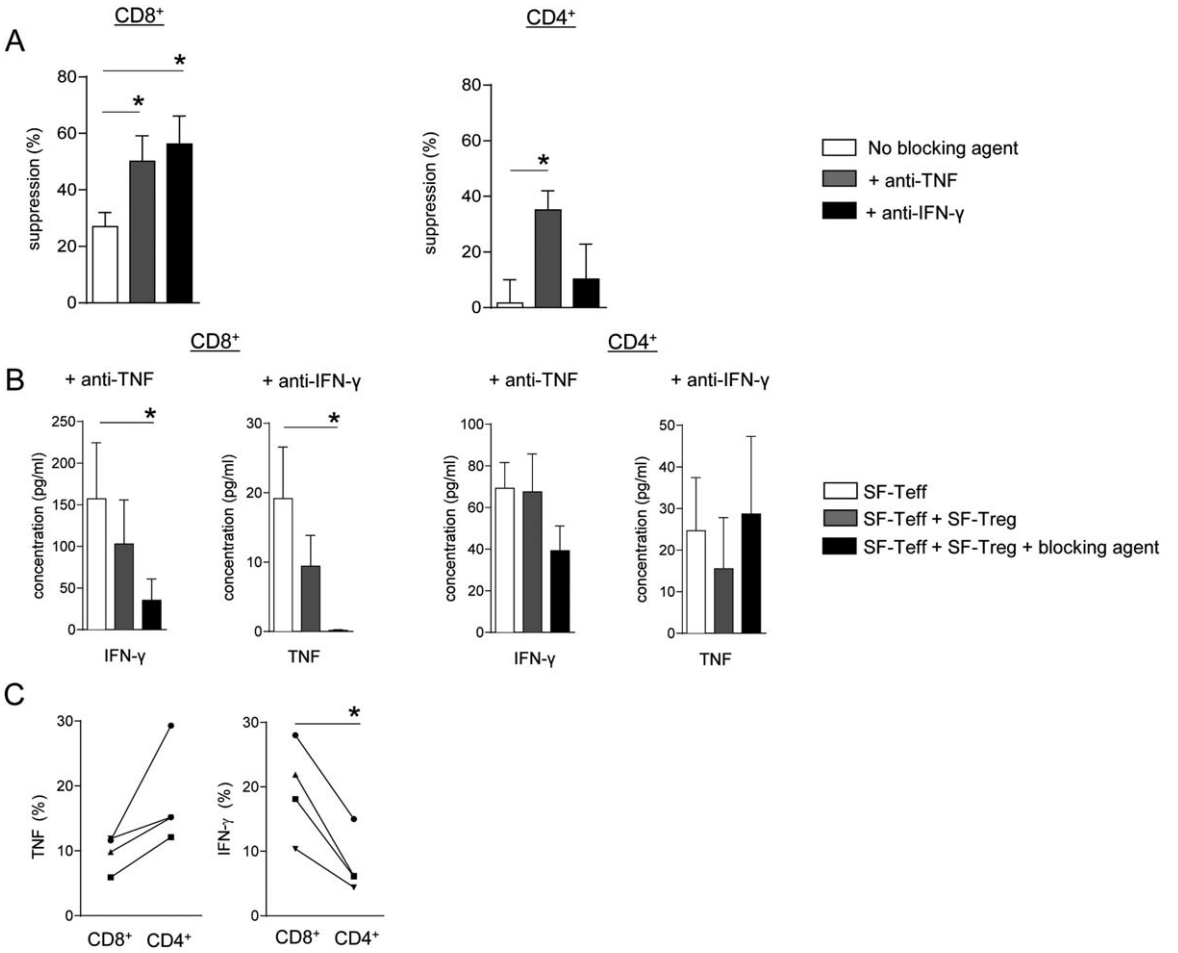

Figure 3. Resistance to suppression of CD8+ Teff cells from synovial fluid (SF) is sustained by autocrine release of tumor necrosis factor (TNF) and interferon-γ (IFNγ), and responsiveness to suppression can be restored by neutralizing these cytokines. **A,** Suppression of SF-derived CD8+ Teff cell proliferation by Treg cells from the same site in the absence and presence of anti-TNF (n = 6 samples) or anti-IFNγ (n = 4 samples). Suppression of CD4+ Teff cells was measured in parallel (n = 7 samples). **B,** Levels of IFNγ and TNF in supernatants of cocultures of CD8+ or CD4+ Teff cells with Treg cells in the presence or absence of anti-TNF and anti-IFNγ, respectively. **C,** Frequency of TNF- and IFNγ-producing CD8+ and CD4+ Teff cells from the SF of patients with juvenile idiopathic arthritis upon stimulation with phorbol myristate acetate/ionomycin. In **A** and **B**, values are the mean ± SEM. In **C**, symbols represent individual samples. $* = P < 0.05$.

library.wiley.com/doi/10.1002/art.39418/abstract). Overall, these data show that CD8+ Teff cells from the site of chronic autoimmune inflammation in JIA patients are intrinsically resistant to suppression, even in the presence of functional Treg cells from PB of JIA patients or healthy controls or in the presence of a potent immunosuppressive cytokine such as TGFβ.

Resistance to suppression of CD8+ Teff cells from SF is self-sustained by TNF and IFNγ, and responsiveness to suppression can be restored by neutralizing either of these cytokines. Given the evidence that both CD8+ and CD4+ Teff cells show intrinsic resistance to suppression, we wondered whether this resistance was sustained by autocrine release of proinflammatory cytokines. TNF has been previously shown to contribute to resistance to suppression of CD4+ Teff cells (6), and TNF blockade was able to restore Teff cell suppression in the presence of APCs (7). IFNγ is a proinflammatory cytokine relevant in autoimmune diseases, and it was clearly detectable in the supernatant of our suppression assay.

In an APC-independent Treg cell–mediated suppression assay with SF-derived cells, coincubation with anti-TNF or anti-IFNγ led to restoration of CD8+ Teff cell suppression of proliferation (Figure 3A). Improvement of suppression was not observed when PB-derived T cells from healthy controls were incubated with blocking antibodies, indicating that this effect is SF T cell specific (data

not shown). However, CD4+ Teff cell proliferation was suppressed in the presence of anti-TNF but not in the presence of anti-IFNγ (Figure 3A). Of note, incubation of CD8+ and CD4+ Teff cells alone with anti-TNF or anti-IFNγ had no effects on cell proliferation (see Supplementary Figures 6A and B, respectively, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39418/abstract).

Coincubation of CD8+ Teff cells with Treg cells and anti-TNF or anti-IFNγ also restored suppression of cytokine production in the culture supernatant (Figure 3B). Coincubation of CD4+ Teff cells with Treg cells and anti-TNF resulted in a slight, yet not statistically significant, improvement in cytokine suppression, whereas anti-IFNγ had no effect (Figure 3B). We then wondered whether the distinct effect of IFNγ blockade on CD8+ and CD4+ Teff cells was due to the different amounts of IFNγ released by the 2 cell populations. Indeed, we found that, unlike TNF, which was produced slightly more by CD4+ Teff cells (Figure 3C), CD8+ Teff cells produced significantly more IFNγ than did CD4+ Teff cells upon brief stimulation with PMA/ionomycin (Figure 3C). Overall, these data show that production of TNF and IFNγ contributes to intrinsic and self-sustained resistance of CD8+ Teff cells to suppression and that blockade of both TNF and IFNγ restores responsiveness to suppression.

DISCUSSION

There has been a large effort in recent years to implement strategies for restoration of immunologic balance between Treg cells and Teff cells in autoimmune diseases (15). Despite the fact that strategies for increasing Treg cell number and function in rheumatic diseases are close to clinical application (for review, see ref. 16), it is still being investigated whether resistance of Teff cells to suppression can be targeted in humans. So far, investigators have focused on the pathogenicity of CD4+ Teff cells, showing their intrinsic resistance to Treg cell–mediated suppression, regardless of the influence of other cell populations (i.e., production of cytokines by APCs or CD8+ T cells, or the ability of Treg cells to suppress proliferation) (8).

In this study, we aimed to determine whether CD8+ Teff cells from the site of autoimmune inflammation in JIA are also intrinsically resistant to suppression and whether autocrine mediators are responsible for this phenotype. We found that Treg cell–mediated suppression of CD8+ Teff cells from SF was impaired regardless of the presence of SF-derived APCs or CD4+ Teff cells. We do not exclude the relevance of those populations in the induction/maintenance of the resistant phenotype, but our data rather indicate that CD8+ Teff cells are able to self-sustain this resistant phenotype. As we were unable to rule out the contribution of SF Treg cells to the observed impairment of Teff cell suppression, we replaced them with Treg cells from the PB of JIA patients or healthy controls (both known to be functional [6]). Our data showed that Teff cell resistance to suppression in the SF occurs regardless of SF-derived Treg cell suppressive abilities. Of note, a slight but consistent improvement of suppression in the presence of Treg cells from the PB was evident, providing indirect evidence that Treg cells residing in the SF might have partial functional defects in an APC-independent setting (as shown in RA [17]).

Further evidence of CD8+ Teff cell intrinsic resistance to suppression was provided by their ability to retain the resistant phenotype in a Treg cell–independent suppression assay, as previously shown for CD4+ Teff cells (6). Resistance to suppression was found to be associated with a memory and activated phenotype of both CD8+ and CD4+ Teff cells; however, further experiments will be needed to prove that this phenotype is responsible for the lack of susceptibility to suppression observed in the SF. Interestingly, we noticed that although Treg cell–independent susceptibility to suppression was similar for both CD8+ and CD4+ Teff cells (suppression of 25% for CD8+ Teff cells versus suppression of 20% for CD4+ Teff cells) (Figure 2C), when Treg cells were present, CD4+ Teff cell resistance to suppression appeared to be higher than that of CD8+ Teff cells (suppression in bead assay of 20% for CD8+ Teff cells versus −5% for CD4+ Teff cells) (Figure 1B). This suggests that SF-derived CD8+ Teff cells are more susceptible to suppression than CD4+ Teff cells and that Treg cells from the SF might have a different modality of suppressing CD8+ and CD4+ Teff cells. However, further studies are needed to elucidate these observations.

We subsequently addressed whether self-produced proinflammatory mediators sustained both CD8+ and CD4+ Teff cell resistance to suppression. Thus, we focused on TNF and IFNγ, both of which are proinflammatory cytokines that are relevant to autoimmune diseases and were clearly detectable in our APC-independent assay. We showed that anti-TNF treatment, which we previously demonstrated to restore Teff cell responsiveness to suppression in the presence of APCs (7), was sufficient to restore CD8+ as well as CD4+ Teff cell suppression in the absence of APCs. However, IFNγ neutralization was effective only in restoring Treg cell–mediated suppression of CD8+, but not CD4+, Teff cells. This is likely to occur due to the major contribution of CD8+ Teff cells to IFNγ release; as CD8+ Teff cells are the major producers of IFNγ, they are probably also more susceptible to its activity.

Previously described mechanisms by which TNF impairs suppression in the SF are hyperphosphorylation of the Akt/protein kinase B (PKB) pathway in Teff cells (6) as well as FoxP3 dephosphorylation in Treg cells (17). IFNγ-induced resistance to suppression is not mediated by the Akt/PKB pathway (data not shown); however, it would be of interest to understand which pathway(s) is involved in the failure of CD8+ Teff cell regulation.

In this study, we showed that CD8+ Teff cells behave differently from CD4+ Teff cells at the site of autoimmune inflammation in JIA and that autocrine IFNγ release selectively sustains the CD8+ Teff cell resistant phenotype. The different behavior of CD8+ Teff cells suggests that this population is endowed with a peculiar effector function, which should be investigated independently of CD4+ Teff cells. This reinforces the idea that new therapeutic approaches targeting the effector cell compartment in autoimmunity should take into account differences within T cell subsets.

In conclusion, we believe that different players in the adaptive immune response (namely, APCs, CD4+ Teff cells, CD8+ Teff cells, and Treg cells) contribute to the defective regulation occurring at the site of chronic autoimmune inflammation, ultimately leading to the breakdown of tolerance. The self-sustained resistance of Teff cells to suppression is a piece of the puzzle with important therapeutic implications. The targeting of Teff cell intrinsic ability to escape regulation is a prerequisite for an effective strategy for the treatment of autoimmune arthritis.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. van Wijk had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Petrelli, Prakken, van Wijk.

Acquisition of data. Petrelli, Scholman.

Analysis and interpretation of data. Petrelli, Wehrens, Vastert, van Wijk.

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