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Cellular Interactions of Synovial Fluid γδ T Cells in Juvenile Idiopathic Arthritis

Anna Bendersky,*1 Victoria Marcu-Malina,*1 Yackov Berkun,† Maya Gerstein,† Meital Nagar,‡ Itamar Goldstein,§ Shai Padeh,† and Ilan Bank*1

The pathogenesis of juvenile idiopathic arthritis (JIA) is thought to involve multiple components of the cellular immune system, including subsets of γδ T cells. In this study, we conducted experiments to define the functional roles of one of the major synovial fluid (SF) T cell subsets, Vγ9Vδ2+ (Vγ9+) T cells, in JIA. We found that as opposed to CD4+ T cells, equally high percentages (∼35%) of Vγ9+ T cells in SF and peripheral blood (PB) produced TNF-α and IFN-γ. Furthermore, stimulation with isopentenyl pyrophosphate (IPP), a metabolite in the mevalonate pathway, which is a specific potent Ag for Vγ9Jy1.2+ T cells, similarly amplified cytokine secretion by SF and PB Vγ9+ T cells. Significantly, the SF subset expressed higher levels of CD69 in situ, suggesting their recent activation. Furthermore, 24-h coculturing with SF-derived fibroblasts enhanced CD69 on the SF > PB Vγ9+ T cells, a phenomenon strongly augmented by zolendronate, a farnesyl pyrophosphate synthase inhibitor that increases endogenous intracellular IPP. Importantly, although Vγ9+ T cell proliferation in response to IPP was significantly lower in SF than PBMC cultures, it could be enhanced by depleting SF CD4+CD25+FOXP3+ cells (regulatory T cells). Furthermore, coculture with the Vγ9+ T cells in medium containing zolendronate or IPP strongly increased SF-derived fibroblasts’ apoptosis. The findings that IPP-responsive proinflammatory synovial Vγ9+ T cells for which proliferation is partly controlled by regulatory T cells can recognize and become activated by SF and then induce their apoptosis suggest their crucial role in the pathogenesis and control of synovial inflammation. The Journal of Immunology, 2012, 188: 000–000.

A subset of T lymphocytes discovered 25 y ago, γδ T cells combine properties of the adaptive and innate immune systems (1, 2). In humans, a major subset of γδ T cells express TCR polypeptides in which the V regions are encoded by rearranged Vγ9 and Vδ2 genes (3). Vγ9Vδ2+ (Vγ9+) T cells can interact with dendritic cells, B cells, and macrophages; kill, in a non-MHC-restricted manner, virally infected and malignantly transformed cells; secrete cytokines and growth factors; and present Ags to CD4+ and CD8+ T cells (4). Most Vγ9+ T cells, which form 50–70% of the circulating γδ T cell subset in healthy humans, use the joining γ 1.2 gene segment rearranged to Vγ9 and uniquely can recognize, in a TCR-dependent manner, small nonpeptidic phosphorylated Ags (including isopentenyl pyrophosphate [IPP]) produced in the mevalonate biosynthetic pathway when their concentration increases due to its dysregulation (5). For example, these phosphorylated moieties may accumulate intracellularly during infection, inflammatory stress, and malignant transformation of epithelial or mesenchymal cells. Furthermore, the same cells can recognize with markedly higher affinity phosphorylated products of the alternative mevalonate pathway in the nonmevalonate (or Rohmer) pathway of isoprenoid synthesis by various microorganisms (6). Recent evidence suggests these substances are recognized by Vγ9Jy1.2Vδ2 T cells in the context of cell-surface membrane-expressed molecules, which may include ectopic F1-ATPase (7).

The mevalonate pathway is critical for the growth of rheumatoid synovial fibroblasts (SFib) because blocking this pathway by inhibiting hydroxy-3-methylglutaryl CoA reductase induces their apoptosis (8). The demonstrated activity of this pathway suggests these substances are recognized by Vγ9Jy1.2Vδ2 T cells, which could potentially activate a subset of synovial Vγ9+ T cells in the joints of patients with autoimmune synovitis (9).

Juvenile idiopathic arthritis (JIA), a clinically heterogeneous group of different disorders characterized by chronic inflammatory arthritis in children, is an important cause of short- and long-term disability (10). Current International League of Associations for Rheumatology classification differentiates subtypes of JIA on the basis of clinical manifestations (oligoarthritis, persistent and extended; rheumatoid factor-positive and -negative polyarthritis; systemic, psoriatic, enthesitis-related, and other undifferentiated arthritis) (11). Although the precise etiology is still unknown, JIA is an autoimmune disease, and T cells are thought to be key players in this process (12). Interestingly, although the Vγ9Jy1.2 gene rearrangement is less commonly expressed among synovial fluid (SF) than peripheral blood (PB) γδ T cells, IPP was recently found to induce expansion of SF Vγ9+ T cell in virtually all forms of JIA (13, 14). However, despite the known proinflammatory potential of Vγ9+ T cells in healthy individuals, higher levels of expansion were associated with clinical remission in these
patients, suggesting that IPP-induced activation of the cells in some unknown manner contributes to control synovial inflammation (13). The experiments described in this paper were undertaken to further delineate functions and cellular interactions of IPP-responsive SF Vγ9 T cells that could be relevant to disease outcomes in these patients.

Materials and Methods

Patients

The study was approved by the Institutional Review Board (Helsinki Committee) of the Chaim Sheba Medical Center, Ramat Gan, Israel. All participating patients were seen in the Pediatric Rheumatology clinic. Patients fulfilled the criteria of the American College of Rheumatology for JIA (11). Informed consent was signed by parents of all participants. All samples of SF and PB were drawn during an acute flare of synovitis, requiring intra-articular corticosteroid injection. The patients participating in the study included individuals with pauciarticular persistent arthritis, with extended JIA, systemic disease, psoriatic arthritis, and polyarthritides. Control samples were drawn from PB of healthy consenting adults.

Isolation of mononuclear cells

PBMC and synovial fluid mononuclear cells (SFMC) were isolated by Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO) density centrifugation as previously described (15).

Fibroblast cultures

Primary fibroblastic lines were obtained from SF removed from knees of patients for therapeutic indications and maintained as previously described (15). In brief, fibroblasts were derived by culturing adherent synovial fluid cells in plastic wells in DMEM-supplemented medium for 48 h followed by removal of all of the nonadherent cells. The adherent cells displayed a fibroblastic morphology and were determined by flow cytometry to express CD90, low levels of the CD49a integrin, to be negative for macrophage (CD14), T cell (CD3), and B cell (CD20) surface markers, and stained positively for collagen type I determined by Sirius red staining (not shown).

Characterization of T cell subsets by flow cytometry

FITC, allophycocyanin, PerCP, or PE-conjugated nonbinding Ig isotype control mAb (BD Pharmingen) or fluorochrome-conjugated mAb to CD4, CD3, CD25 (BD Pharmingen), or the human Vγ9, Vδ1, and Vδ2 gene products (ImmunoTech) were added to a resuspended pellet of 2 × 10^6 mononuclear cells (MC) for 45 min on ice (15). Cells were then washed in PBS (pH 7.4; Life Technologies, Invitrogen). The anti-human FOXP3 mAb (PECH101) staining kit was purchased from eBioscience and used as per the manufacturer’s instructions. Lymphocytes were gated according to typical forward and side scatter plots using an FACS flow cytometer (BD Biosciences, Mountain View, CA), and analysis of the gated cells was performed.

Purification of T cell populations

CD25^+ cells were isolated using the MACS system (Miltenyi Biotec) according to the manufacturer’s instructions. SFMC were also stained with FITC-conjugated anti-CD4 and/or PE-conjugated anti-CD25 mAbs (BD Pharmingen) for 45 min on ice. PBMC activated with IPP were immunodepleted using mAb to CD4 and CD8 to obtain >90% purified Vγ9 T cells. Cells were then washed twice with MACS buffer (0.2 mM EDTA and 0.5% BSA in PBS). Subsequently, cells were incubated with anti-mouse IgG microbeads (Miltenyi Biotec) for 15 min at 4–8°C and passed through MACS columns. Cells retained in the columns were eluted and used in experiments after confirmation of purity by FACS.

In vitro stimulation

PBMC or SFMC (1 × 10^6 cells/ml) were cultured in 96-well, round-bottom tissue-culture plates (Costar) in 200 μl RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 μg/ml penicillin-streptomycin solution, and 100 IU/ml recombinant human IL-2 (rHL-2; Boehringer Mannheim) and stimulated by addition of IPP (Sigma-Aldrich) as indicated in the respective experiments. An αβ TCR selective superantigen, toxic shock syndrome toxin-1 (TSST1; Sigma-Aldrich) at 10 ng/ml, or rHL-2 alone was added as indicated. Medium was replaced on days 3 and 7 of culture. All cultures were maintained for 10 d except where indicated, at which time they were used for experiments.

Proliferation assay

T cell proliferation was assessed, as previously described, using FACS-based analysis of serial halving of the vital dye CFSE (16). Briefly, up to 1 × 10^6 cells were suspended in 1 ml RPMI 1640 medium and then pulsed with 2.5 μM CFSE (Molecular Probes) for 10 min (37°C) with constant shaking. Subsequently, the CFSE was quenched with supplemented RPMI 1640 medium, and then the cells were washed twice with large volumes of medium. The labeled cells were stimulated and cultured, as indicated, and at the end of culture were analyzed for CFSE dilution.

Cytokine detection

For intracellular cytokine detection, the T cells were activated with 20 ng/ml PMA and 0.8 μM monomycin (Sigma-Aldrich) in the presence of 2 μg/ml brefeldin A (Golgistop; eBioscience) for 4.5 h. Subsequently, the cells were fixed and stained with anti-Vγ9-PE and CD4-PE and washed twice in PBS. Cells were fixed and permeabilized using fixation and permeabilization buffers (eBioscience), then stained in Flow Cytometry Staining buffer (eBioscience) with anti-TNF-α, anti–IL-17, and anti–IFN-γ–allophycocyanin. IFN-γ detection in supernatants was done using the ELISA max Deluxe Sets (BioLegend, San Diego, CA) according to the manufacturer’s instructions.

Fibroblast/T cell cocultures

A total of 100 μl (2 × 10^5 cell/ml) Stbl, resuspended in DMEM-supplemented medium, were placed in 48-well flat-bottom plates (Costar). After 1 h, during which time fibroblasts formed a homogeneous monolayer, effector T cells resuspended in RPMI 1640-supplemented medium were added at the indicated concentration (usually 2 × 10^6 cells/ml). Cocultures were placed in the humidified 5% CO_2 incubator at 37°C for 48 h. To evaluate fibroblast growth, nonadherent cells were decanted and adherent cells fixed in 70% ethanol for 10 min. Cells were then stained with 1% (w/v) crystal violet solution for 20 min, followed by extensive washing with water, and were evaluated by inverted microscopic inspection. To determine apoptosis, fibroblasts were stained with CFSE prior to plating. After coincubation with the designated population of T cells for 48 h, adherent cells in each well were treated with trypsin-EDTA solution for 3 min (2×) and stained with anti–CD49a-PE and anti–CD90-FITC and CD4-PE and washed in PBS. The cells were spun down at 350 × g, stained with Annexin V (BD Pharmingen), and analyzed on an FACScan flow cytometer (BD Biosciences). Analysis of CFSE+ cells was performed and the percentage of Annexin V+ cells recorded.

Suppression assay

Regulatory T cells (Treg), isolated from SFMC or PBMC, were tested for their ability to suppress the proliferation of autologous CD25^+ Vγ9^+ T cells using methods similar to those previously described (16). Briefly, CFSE-labeled CD25^+ SFMC or PBMC at 1 × 10^5 cells/well were plated into 96-well plates (Costar) and activated by either anti-CD3 mAbs (clone OKT3; 2.5 μg/ml), IPP (2 μg/ml), or both reagents in the presence of graded amounts of CD25^+ T cells. Five days later, the T cells were harvested and analyzed by FACS. Vγ9^+ T cells proliferation was determined using the proliferation platform of FlowJo 7.2.5 (Tree Star) to calculate the division index (DI) and draw a fit model of generations onto the CFSE dilution histograms. The DI reflects the average number of cell divisions that the cells underwent and is considered a good objective value to compare the rate of proliferation from sample to sample. Percent suppression was calculated for each well using the following formula: 100 × (DI in the absence of CD25^+ T cells – DI with added CD25^+ T cells)/DI in the absence of CD25^+ T cells.

Statistical analysis

Student t tests (Prism software; GraphPad) were used to analyze the significance of differences between groups.

Results

Cytokine production by SF Vγ9^+ T cells and effect of IPP stimulation

To determine the cytokine profile of SF Vγ9^+ T cells, relevant to synovial inflammation, freshly isolated SF and PBMC from 12 patients with JIA were activated with PMA and ionomycin in the presence of a Golgi-transport inhibitor and then analyzed for intracellular levels of IFN-γ, TNF-α, and IL-17. We found that SF CD4^+ T cells were significantly enriched for TNF-α (21.7 ±
5.0%) and IFN-γ–producing (13.7 ± 4.4%) cells compared with their PB counterparts (8.7 ± 4.1%; p = 0.05 and 2.2 ± 1.1%; p = 0.017, respectively). By contrast, a higher percentage of Vγ9+ T cells produced TNF-α and IFN-γ both in the SF compartment and PBMC (Fig. 1Ai, 1Aii). Thus, the mean percentage of IFN-γ–expressing Vγ9+ T cells in PB, but not SF, exceeded that of the CD4+ subset (p = 0.008). In addition, we tested the cytokine production by another γδ T cell subset, Vδ1, that is equally represented in the synovium of JIA patients (13). High percentages of SF Vδ1+ T cells secreted IFN-γ and TNF-α (Fig. 1Aiii). IL-17 production within all three cell subsets, CD4+, Vγ9+, and Vδ1+, from either PB or SF was restricted to a small percentage of the cells (<10%), with the exception of the PB Vδ1+ subset (∼30%).

Next, the contribution of the IPP-responsive Vγ9+ T cell subset to cytokine production was assessed. Thus, SFMC and PBMC from patients (n = 10) were cultured in vitro for 5 d in medium containing IPP (2 μg/ml), medium alone or with TSST1 (10 ng/ml) as a control stimulus for CD4 cells, followed by activation with PMA and ionomycin. As found in freshly isolated cells, the proportion of cultured SF CD4+ T cells producing TNF-α and IFN-γ was significantly increased compared with cultured PB CD4+ T cells (27.4 ± 4.7% versus 10.64 ± 1.7%, p = 0.0028; and 9.3 ± 2.5% versus 1.4 ± 0.9%, p = 0.006, respectively). The cytokine production was unaffected by IPP, whereas stimulation with TSST1 induced significantly higher percentages of IFN-γ– but not TNF-α–producing CD4+ cells in SF and PB compared with medium alone or IPP (Fig. 1Bii; p = 0.0002 and 0.0001, respectively). In contrast, the percentages of cytokine-secreting Vγ9+ T cells in cultures of PB and SF were rather similar: 34.2 ± 5.2% versus 27.5 ± 4.5% (p = 0.3) for TNF-α and 16.6 ± 5.9% versus 24.4 ± 5.1% (p = 0.3) for IFN-γ, and generally higher compared with CD4+ T cells. Furthermore, preincubation with IPP, but not TSST1, significantly increased the percentage of IFN-γ+, TNF-α+, and IL-17–producing Vγ9+ T cells in both PB and SFMC cultures (Fig. 1Bii). Notably, the relatively high levels of TNF-α and IFN-γ production by cultured Vδ1+ T cells were not influenced by activation with IPP or TSST1 and did not significantly differ between SF and PB subsets, whereas their production of IL-17 was very low in vitro (Fig. 1Biii). These in vitro patterns of response of the CD4 and Vγ9+ T cells from JIA patients partly

![Figure 1](http://www.jimmunol.org/downloads/13082017/13082017-fig1.jpg)
FIGURE 2. CD69 and CD25 expression in situ, after activation with IPP and in response to SF. (A) SF and PBMC from JIA patients were stained directly with mAb to CD3, V<sub>γ</sub>9, CD69, and CD25 immediately after isolation (0 h) or following 24 h incubation with 100 IU/ml rhIL-2 and 2 μg/ml of IPP. Shown are representative dot plots of CD3 and V<sub>γ</sub>9 staining (left panel) and histograms of CD69 and CD25 staining of the CD3<sup>+</sup>V<sub>γ</sub>9<sup>+</sup> population from SF (dashed line) or PB (dotted line) as compared with negative control isotype staining (gray fill) (top right panel). Data summary from JIA patients (n = 7 for 0 h, n = 3 for 24 h) of CD69 and CD25 expression is shown in scatter plots and bar diagram below the histogram (bottom right panel). The p values for differences between means at 0 h are indicated. The p values for comparison with values in medium alone are *p < 0.01, **p < 0.001. (B–D) Isolated SF or PBMC were incubated alone, with synovial fibroblast (Syn-Fib), or with foreskin fibroblast (FS11) in presence or absence of zoledronate (Zol; 2 μM) for 24 h. Following incubation, cells were stained with CD3-, V<sub>γ</sub>9-, and CD69-specific mAb, and supernatants were analyzed for IFN-γ secretion with ELISA. (B) Bars represent MFI ± 1 SEM of CD69 staining of PB (hatched bars) or SFMC gated on CD3<sup>+</sup> and either V<sub>γ</sub>9<sup>+</sup> (white bars) or V<sub>γ</sub>9<sup>-</sup> (gray bars) populations (n = 4). Mean of CD69 MFI level expressed by cells in the medium was compared with that on stimulated cells by Student t test (Figure legend continues).
resembled those of these subsets in PBMC isolated from healthy adults (Fig. 1Biv–1Bvi). Thus, IPP specifically and significantly increased TNF-α production by cultured Vγ9δ T cells from healthy donors but not by CD4+ or V61+ T cells. In contrast, TSST1 increased this cytokine production only by cultured CD4+ T cells. The effect of IPP on IFN-γ production in the healthy donor-derived Vγ9δ T cells was not significant, possibly due to the high percent of spontaneously producing IFN-γ cells in these donors.

Further, to analyze the contribution of Vγ9δ T cells to the cytokine pool produced by SFMC, we compared cytokine production by Vγ9-depleted SFMC and Vγ9δ (+90%) T cells from a donor whose SFMC contained a high percentage (18.7%) of Vγ9δ T cells after triggering with IPP, TSST1, or PMA and ionomycin for 72 h (Fig. 1C, 1D). Very low or absent levels of cytokines were produced in medium alone, whereas a high concentration of IFN-γ was secreted by unseparated SFMC, Vγ9δ, and Vγ9δ SFMC stimulated with PMA and ionomycin. Depletion of the Vγ9δ T cells almost abolished production of IFN-γ and TNF-α, whereas the isolated Vγ9δ T cells produced notably higher amounts of TNF-α than unseparated SFMC and Vγ9δ SFMC in response to IPP. By contrast, TSST1-induced cytokine production was mainly contributed by Vγ9+ cells.

These results suggest that Vγ9δ T cells, specifically, of JIA patients can strongly contribute to the repertoire of TNF-α, IFN-γ, and, to a lesser degree, IL-17 produced systemically and in the synovium upon activation with IPP.

**Activation of SF Vγ9+ T cells in vivo and in vitro**

To test the hypothesis that SF Vγ9δ T cells may be responding to subset-specific Ags at the site of inflammation, we determined whether they express an activated phenotype consistent with a recent encounter with IPP. First, we examined CD69 and CD25, cell-surface markers of recent activation, on the Vγ9δ T cells in PB and SF of JIA patients. We found that SF Vγ9δ T cells expressed significantly higher levels of CD69 (mean fluorescence intensity [MFI] 89.5 ± 15.3) than PB Vγ9δ T cells (MFI 37.5 ± 3.9; p = 0.0065), whereas both subsets express only low levels of CD25 in situ (Fig. 2A, 2A, 0 h). In vitro stimulation with IPP for 24 h induced significantly higher levels of CD69 and CD25 on the two populations of Vγ9δ T cells, as compared with cells cultured in IL-2 medium alone (Fig. 2A, 24 h). These results suggest that in the inflamed tissue, SF Vγ9δ T cells express at least one of the hallmarks consistent with that of cells recently activated by IPP (i.e., CD69hiCD25hi).

Monocytes and dendritic cells are known to activate PB Vγ9δ T cells when they are induced to express high levels of IPP, for example, by zolendronate, an inhibitor of farnesyl pyrophosphate synthase (17, 18). To determine whether other cellular components of the synovium also play a role in activating SF Vγ9δ T cells, we focused on the role of SFib, which are critically dependent upon the mevalonate pathway, suggesting that they may continuously produce the Vγ9 Ag IPP (8).

SFMC were isolated from four JIA patients and cocultured for 24 h either alone or with zolendronate in the presence or absence of SFib or foreskin fibroblasts and analyzed for CD69 expression. We found that brief ex vivo treatment of PBMC and SFMC with zolendronate enhanced CD69 expression specifically on Vγ9δ T cells but not on Vγ9+ populations of T cells (Fig. 2C). However, coculturing of the PBMC and SFMC with SFib, even in the absence of zolendronate, enhanced CD69 expression on SF Vγ9δ > Vγ9+ T cells, but not on PB-derived cells. Coculturing with foreskin fibroblasts alone, by contrast, did not induce significant changes in CD69 levels within these T cell populations. Importantly, treating the cocultures containing either SF or foreskin fibroblasts with zolendronate further enhanced CD69 expression on both SF and PB Vγ9δ T cells specifically. Polyclonal stimulation with anti-CD3, as predicted, induced nonspecific CD69 upregulation on the majority of PB- and SF-derived T cells (data not shown).

Next, we tested whether contact with SFib will induce cytokine expression by SF- and PB-derived T cells. We found that IFN-γ secretion was not increased in the presence of SFib or foreskin fibroblasts alone. Of note, SFMC spontaneously secreted significantly higher levels of IFN-γ compared with PBMC, and zolendronate alone enhanced IFN-γ secretion only in the PBMC. Strikingly, IFN-γ secretion could be induced most significantly in both SFMC and PBMC by coculturing with SF or foreskin fibroblasts coupled with zolendronate treatment (Fig. 2D). In general, a similar pattern of induction of TNF-α secretion was observed, although the concentration of this cytokine was much lower (Fig. 2D). Furthermore, depletion of the Vγ9δ T cells, but not sham depletion, prior to the coculture with SFib, completely abrogated the significant increase in IFN-γ secretion by Vγ9δ T cells following their culture with zolendronate-stimulated fibroblasts (Fig. 2E).

These results show that contact with SF fibroblasts alone can specifically activate SF Vγ9δ T cells to express CD69 in vitro, whereas induction of significantly enhanced IFN-γ (and to a lesser degree of TNF-α) secretion requires an additional stimulus that can be provided by zolendronate.

**IPP-dependent SFib apoptosis induced by SFMC Vγ9δ T cells**

SFib contribute to destructive arthritis by invasive proliferation (19) and are thus putative therapeutics targets in inflammatory arthritis (20). IPP-activated PB Vγ9δ T cells from healthy individuals and scleroderma patients can disrupt fibroblast monolayers in vitro and induce fibroblast apoptosis partly mediated by TNF-α (15). Our current results indicated that TNF-α production by SF Vγ9δ T cells can be induced by IPP activation (Fig. 1) and that contact per se with SFib can also activate SF Vγ9δ T cells (Fig. 2C, 2D). Thus, we next asked whether IPP-activated Vγ9δ T cells SFMC could induce SFib apoptosis. To address this, we first activated SFMC for 10 d in culture medium, with IPP (2 μg/ml), or with TSST1 (to activate and to enrich the CD8 TCR+ SF T cell population). The FACS analysis of the T cell cultures showed, as expected, enrichment for Vγ9δ T cells only in IPP-stimulated cultures (Fig. 3A). Subsequently, the various T cells were added to confluent monolayers of fibroblasts derived from the synovium of JIA patients, and control monolayers of fibroblasts were cultured alone. Microscopic examination, at 48 h, revealed that SF-derived fibroblast monolayers cocultured with SFMC expanded in

\( *p < 0.05, **p < 0.01 \). (C and D) Bars represent mean ± 1 SEM of IFN-γ and TNF-α in supernatants of cultures \((n = 4)\). Statistical evaluation of the differences between cells in medium alone and with the indicated stimulus for T cell population derived from SF or PB were calculated \((p < 0.05, **p < 0.01)\). Significant differences between mean concentrations of SF and PB are also indicated \((p < 0.05)\). (E) IFN-γ in the supernatant of unseparated SFMC (Total SF), SFMC depleted of Vγ9δ T cells (SF Vγ9δ), or Vγ9δ T cells in the SFMC (SF Vγ9δ) after culture in the presence of Zol, SFib (Syn-Fib), or both. **p < 0.001 for comparison of indicated means \((n = 3)\).
FIGURE 3. SF Vγ9+ T cell-mediated apoptosis of Sfib is dependent on exogenous and endogenously produced IPP. (A) Photomicrographs (original magnification ×200) of 48 h confluent cultures of synovial fluid fibroblasts in medium alone or with 2 μg/ml IPP without added SFMC (None) or with (cell/cell ratio of 10:1) SFMC from a JIA patient that had been previously cultured for 10 d in medium plus 100 IU rhIL-2 alone (SF T cells), with IPP (IPP-act SF T cells), or with TSST1 (TSST1-act SF T cells) (left panel). FACS dot plots of the respective SFMC cultures stained with mAb to Vγ9 and CD3 are shown in the right panel, and percent of CD3+Vγ9+ and Vγ9+ T cells is indicated. (B) FACS dot plots of CFSE-labeled synovial-derived fibroblasts (Fib) that were cultured alone for 48 h, with IPP-activated SF T cells, or with TSST1-activated SF T cells as indicated in (A), then harvested and stained with PE-labeled Annexin-V. Percent of Annexin-V+/CFSE+ cells is indicated. (C) Bars represent mean ± 1 SEM of percent of apoptotic Sfib following coculture for 48 h at a 1:10 cell/cell ratio with SF or PBMC from three JIA patients that were prestimulated with IPP for 10 d. The p values for comparison with adjacent bars to the left are indicated (*p < 0.01, **p < 0.001). (D) CFSE-labeled Sfib were cocultured with >90% purified Vγ9+ T cells from PBMC of healthy individuals (n = 5) at the indicated ratios for 48 h and percent of apoptotic cells recorded as in (B). (E) Bars represent mean percent of apoptotic Sfib ± 1 SEM following 48 h of coculture all in presence of zoledronate (2 μM) at a 1:10 cell/cell ratio with Vγ9+ T cells >90% purified from PBMC (γδ T cells) of two healthy donors that had been stimulated for 10 d with IPP plus IL-2 or with the same PBMC that had been stimulated with TSST1 plus IL-2 (αβ T cells).
Proliferative response to IPP. (A) Shown is number of cells per milliliter ± 1 SEM of CD3^+ Vγ9^+ T cells of paired PB and SFMC of 14 JIA patients in freshly isolated samples (time 0) and after 10-d culture in vitro with increasing doses of IPP plus 100 IU/ml rhIL-2. The p values for comparison between the mean in PB and SF at each concentration of IPP are indicated (**p < 0.01, ***p < 0.001). (B) Representative dot plot FACS analysis of cultured SF and PBMC from a JIA patient stained with mAb to Vγ9 and CD3 after stimulation with the indicated concentration of IPP. Percent of CD3^+ Vγ9^+ T cells in each culture is indicated in the top right quadrant. (C) CFSE-labeled PB or SFMC cultures were triggered with rhIL-2 (100 U/ml) alone (Med), or with 2 μg/ml IPP, or 10 ng/ml TSST1. Five days later, mean percent ± 1 SD of dividing cells within indicated subsets from flow cytometric analysis was calculated. Number of experiments for each evaluation is indicated in parentheses. Statistical significance is shown as *p < 0.05, **p < 0.01, ***p < 0.001, respectively, for comparison with medium alone. (D) FACS analysis of CFSE-loaded SFMC gated on Vγ9^+ T cells derived from four different JIA patients (Pt) after 5 d in culture with 2 μg/ml IPP and 100 IU rhIL-2. Each peak to the left of the first on the right indicates a round of cell division corresponding to the dilution of CFSE. Percent of dividing cells within the Vγ9^+ subset is indicated in the top left corner.

IPP-induced proliferation of SF-derived Vγ9^+ T cells is lower than that of PB-derived Vγ9^+ T cells

We next assessed the proliferative potential of IPP-responsive SF Vγ9^+ T cells relative to their PB counterparts. The absolute number and percentage of Vγ9^+ T cells in SF or PB in freshly isolated samples was rather similar, and moreover, their expansion in control medium was minimal upon culturing with IL-2 alone (Fig. 4A, 4B; p = 0.9). However, the IPP dose-dependent expansion of Vγ9^+ T cells was significantly lower in SF versus PBMC cultures, both numerically and as a percentage of the cultured T cells (Fig. 4A, 4B, respectively). Using the CFSE-dilution assay we further demonstrated that the cell division rate of Vγ9^+ T cells from SF was also significantly lower compared with those from PB (31.0 ± 19.7% versus 63.5 ± 12.5%; p < 0.003; Fig. 4C). Notably, as a control for the specificity of IPP, the proliferation of SF CD4^+ T cells was induced by TCRβ-specific superantigen TSST1 but not IPP (Fig. 4C). Interestingly, the rate of IPP-induced proliferation of the SF Vγ9^+ T cells was highly variable among different patients, and some exhibited quite strong proliferative responses (Fig. 4D; 7.8–42.3%; n = 4).

These results indicated that proliferation of SF Vγ9^+ T cells in response to IPP in vitro is significantly lower than that of their PBMC counterparts in this cohort of patients, which may explain...
FIGURE 5. CD25⁺CD4⁺ T cells (Treg) decrease SF Vγ9⁺ IPP-induced proliferation. (A) Dot plots from FACS analysis of untouched SFMC (SF total), SFMC immunomagnetically depleted of the CD25⁺ cells (CD25⁻), and CD25⁺ cells retrieved from the columns (CD25⁺), subsequently stained with mAb to CD25 and CD4 (top panel). The same populations were permeabilized and stained with mAb to FOXP3 and anti-CD25 (bottom panel). Percentages of cells expressing both markers are indicated in each dot plot. (B) CFSE-loaded SFMC or PBMC were cultured for 5 d in medium with rhIL-2 (100 IU/ml) and IPP (2 μg/ml). Prior to incubation, SFMC were subjected to immunomagnetic depletion of CD25⁺ cells (SF CD25⁻) or using nonspecific murine IgG [SF (sham depletion)]. Shown is proliferation FACS analysis of cells gated on Vγ9⁺ population. Each peak to the left of the first peak on the right represents a round of cell division. Percentages of the dividing Vγ9⁺ T cells are indicated in bold. (C) Dose-response curves (graphs I–III corresponding to non-depleted, IgG-depleted, and CD25-depleted SFMC, respectively) of percent Vγ9⁺/total CD3⁺ T cells in 10-d in vitro-stimulated cultures with indicated concentrations of IPP. (D) Percent ± 1 SEM of divided Vγ9⁺ T cells (mean ± 1 SEM; n = 7) in SFMC cultures stimulated with 100 IU/ml of rhIL-2 only (black bars) or 2 μg/ml of IPP (gray bars) for 5 d. Prior to stimulation, SFMC were either depleted of CD25⁺ cells (SF w/o) (Figure legend continues)
their lower capacity to induce fibroblast apoptosis as observed in the previous set of experiments (Fig. 3C).

**Influence of CD4+CD25+FOXP3+ Treg on IPP-responsive Vγ9+ T cells**

Treg have been demonstrated in SF of JIA patients, suggesting they may regulate SF Vγ9+ T cell proliferation (21, 22). To probe whether Treg from SF indeed suppress IPP-induced activation of SF Vγ9+ T cells in JIA patients, we depleted, using CD25+ microbeads (MACS cell separation system), the majority of CD4+CD25hiFOXP3+ T cells from the SFMC population. As seen in the representative example shown in Fig. 5A, CD25+ populations isolated in these experiments were highly enriched for FOXP3+ cells (85%), contrasting with 18 and 9% FOXP3+ in the unseparated and CD25-depleted SFMC, respectively. We found that following IPP stimulation, depletion of CD25+ Treg augmented the proliferation of Vγ9+ T cells in SFMC cultures compared with sham depletion and untouched SFMC, as assessed by FACS analysis of CFSE dilution within the Vγ9+CD3+ events gate (Fig. 5B; 22.8, 11.5, and 7.8%, respectively). Nevertheless, the proliferation of CD25+ SFMC did not reach the rate observed in PB Vγ9+ T cells. Likewise, in another JIA patient, we found that CD25+ T cell depletion also augmented proliferation in an IPP dose-dependent manner (Fig. 5C) as compared with sham depletion or untouched SFMC cultures. In addition, the cumulative data from analogous experiments repeated in seven different JIA patients show significant increase of the proliferation of Vγ9+ T cells in SFMC cultures in which CD25+ cells were depleted compared with sham depletion (49.4 ± 8.1% versus 37.2 ± 6.8%; p < 0.002; Fig. 5D). We further found that CD25+ T cell depletion significantly increased IFN-γ+ (CD25-depleted, 68.8 ± 8.5% versus nondepleted, 59.6 ± 10.8%; p < 0.02), but not TNF-α+ (46.9 ± 18 versus 44.9 ± 9.7% respectively; p = 0.77) Vγ9+ T cells in the 5-d IPP cultures (data not shown). To confirm that SF Treg actively suppress IPP-induced SF Vγ9+ T cell proliferation, CD25+ SFMC (>70% FOXP3+) were immunomagnetically separated from SFMC. As a positive control for these experiments, we used PBMC from a healthy adult. These Treg were then added back to CFSE-loaded CD25-depleted SFMC (Tall) at different Treg to CD25-SFMC ratios and cultured in medium containing a low concentration of IL-2 alone or with added IPP, anti-CD3 mAbs, or IPP plus anti-CD3 mAbs. FACS analysis of CFSE dilution in Vγ9-stained cells after 5 d of culture revealed a strong and dose-dependent suppressive effect of the Treg on proliferation of SF Vγ9+ T cells. We found that Treg could suppress the proliferation of Vγ9+ T cells regardless of the stimuli employed. Interestingly, lower ratios of SF-Treg/Tall were required to achieve ~60% suppression of Ag-specific (IPP) versus polyclonal-induced (anti-CD3) Vγ9+ T cell activation (1:8 versus 1:2, respectively; Fig. 5E). Treg from PB of healthy controls also suppressed IPP-induced proliferation of Vγ9+ T cells, but required a higher Treg/Tall ratio of 1:1 to achieve ~60% suppression. The results of these experiments are consistent with the hypothesis that in JIA patients with a reduced in vitro response of Vγ9+ T cell to IPP, SF-Treg may be playing a critical suppressive role.

**Discussion**

This study shows for the first time, to our knowledge, that Vγ9+ T cells in SF of JIA patients consist primarily of a functionally important population of proinflammatory cytokine-secreting cells, including IPP-responsive cells, that are largely in an activated state in vivo. Our data further suggest that Sfib can potently drive their activation, at least in vitro, in a manner that produces a functional outcome (e.g., IFN-γ secretion), especially in the presence of a reagent that induces cellular accumulation of IPP, a potent Ag for a subset of human Vγ9+ T cells (e.g., zoleodronate). Furthermore, SF Vγ9+ T cells can induce apoptosis of Sfib that are exposed to a reagent, zoleodronate (7). These latter findings suggest an immunological mechanism that may underlie the recently described correlation between recovery from JIA and higher levels of IPP-responsive synovial Vγ9+ T cells (13).

Current paradigms of immunopathogenesis of JIA envision autoreactive αβ T cells responding to a limited repertoire of autoantigens in the joint, initiating the inflammatory response (10). In full-blown clinical disease, the immune response may expand to include multiple autoantigens presented by dendritic cells to autoreactive CD4+ T cells that subsequently activate Sfib and innate immune cells, all producing multiple proinflammatory cytokines (12). It has been shown that autoreactive effector CD4+ T cells can be suppressed by CD4+CD25highFOXP3+ T cells, theoretically providing a mechanism to terminate such pathogenic activation.

In this study, we focus on the SF Vγ9+γδ T cell subset and show that a high proportion of these cells secrete TNF-α and IFN-γ and a lower percentage IL-17, an effector function that increased upon activation by IPP presented either by conventional APCs within the SFMC population or by adherent fibroblasts. In this respect, TNF-α is a central mediator of multiple aspects of synovial inflammation that can activate fibroblasts, osteoclasts, B, and T cells and is an established therapeutic target in JIA (23). IFN-γ may also play a pathogenic role by activating macrophages, whereas IL-17 increases vascularization and induces influx of neutrophils to drive inflammation (24, 25). Thus, each of the cytokines shown in this study to be abundantly produced by SF Vγ9+ T cells, and in particular by the IPP-responsive subset, could potentially contribute to the inflammatory process in JIA.

By contrast, other well-described functions of the same cytokines could paradoxically dampen the inflammatory response. For example, contact-dependent induction of Sfib apoptosis by IPP-activated Vγ9+ T cells is partly TNF-α dependent, as it can be reduced by blocking mAbs to TNF-α (15). This TNF-mediated effect could potentially reduce chronic inflammation and cartilage destruction, thus producing a protective effect in arthritis. Likewise, IFN-γ can enhance Treg function, inhibit differentiation of Th17 cells, and attenuate tissue damage (24). These effects of TNF-α and IFN-γ suggest that under certain conditions, a proinflammatory cytokine could play a beneficial, rather than a pathogenic, role in JIA. The recent finding of a clinical correlation between recovery from JIA and the number SF Vγ9+ T cells and their ability to expand in response to IPP suggests that the cytokines produced by activated Vγ9+ T cells in the synovium of JIA may paradoxically contribute to the recovery of the immune response.
patients may actually tilt the balance toward a beneficial outcome (13).

Our data show that both Vγ9+ T cells in synovium and in PB are enriched for cells with the immediate effector capacity to produce various cytokines, whereas SF CD4+ T cells were selectively enriched for immediate effectors relative to their PB counterparts (Fig. 1). This finding is consistent with the concept that as opposed to SF CD4+ T cells that are selected by site-specific autoantigens, Vγ9+ T cells recognize ubiquitously expressed phosphorylated autoantigens, such as IPP (26). However, SF Vγ9+ T cells in the synovium were unique in expressing a phenotype consistent with recent activation (e.g., expressing a higher level of CD69 than their PB counterparts). This suggests that SF Vγ9+ T cells have encountered autoantigens more recently in situ. Moreover, SF-resident Vγ9+ T cells, as opposed to their PB counterparts, immediately upregulated CD69 when they contacted SF, whereas both populations could be further activated in the presence of zolodenate. Thus, we postulate that SF Vγ9+ T cells may have been primed in vivo by exposure to stressed cells expressing high levels of IPP or by exogenous sources of phosphorylated Ags (e.g., those produced by bacterial nonmevalonate pathways) and are thus able to respond to the lower levels of IPP that may be expressed by untreated Sfib. The mechanisms involved in this heightened level of responsiveness could include elevated expression of costimulatory molecules as well as the selection of TCR with higher affinity for IPP in the synovium-residing population. It is also possible that Ags other than IPP expressed by SF are being recognized by oligoclonal TCRs that are more abundant in the SF Vγ9+ T cell population, but infrequent in PB. Obviously, further experiments are required to address this hypothesis.

In some patients, which may include those with relatively weak proliferative responses, the Vγ9+ subset localizing to the SF is under tight control of SF CD4+ Treg (Fig. 5). However, in the majority of patients with a substantial proliferative response to IPP, Treg control was significant but marginal. We recently reported that higher IPP responses, suggesting that Treg are less efficient in this regard, are associated with better outcomes of JIA (13). The current analysis suggests that strong IPP-induced proliferation driven by endogenous upregulation of this metabolite could lead to local increases of Vγ9+ T cells in the synovial microenvironment. This activation might suffice to induce apoptosis of certain Sfib. This IPP-responsive Vγ9+ T cell-mediated effect could play a critical negative-feedback role in ameliorating disease in JIA patients. This could be more vital in patients with less efficient Treg because fibroblasts contribute to inflammatory cytokines and cartilage destruction (19). To test this postulation, multivariate correlations between the number and function of Treg relevant to autoreactive SF CD4+ T cells and the IPP-dependent responses of SF Vγ9+ T cells and disease outcomes will be required to dissect the relative importance of each mechanism in the different subgroups of JIA patients. Interestingly in this regard, Macaubas et al. (27) recently reported a downregulation of PB γδ T cells, but not of NK cells, during acute exacerbations of systemic JIA specifically, suggesting a unique and specific role of γδ T cells during flares of disease in this particular subset of JIA patients. Although we have not dissected the functional role of Vγ9+ T cells in this subset in particular, our previous results (13) indicate that Vγ9+ T cells in the SF of systemic JIA patients can respond to IPP. Taken together with the data of Macaubas et al. (27), it could be postulated that during acute flares of systemic JIA, PB Vγ9+ T cells migrate to synovium, where they secrete TNF-α and IFN-γ, thus contributing to local inflammation. Notably, highly efficient induction of apoptosis of fibroblasts by Vγ9+ T cells in vitro required exogenous IPP, suggesting that in vivo Sfib that present low levels of IPP will likely escape Vγ9+ T cell-mediated apoptosis. In contrast, treatment of fibroblasts with zolodenate strongly enhanced SF Vγ9+ T cell activation and antifibroblast function. Thus, we envision that when IPP is over-produced by proliferating Sfib (e.g., in the inflamed synovium), the IPP-reactive Vγ9+ T cells are activated to express CD69 and induce their apoptosis. Interestingly, the mevalonate pathway is required for matrix metalloproteinase-9–mediated invasive behavior of murine fibroblasts, suggesting that high levels of IPP may be a marker of the most invasive SF (28). Further studies of the in vivo conditions that enhance IPP production by Sfib and their interaction with PB Vγ9+ T cells may further our understanding of mechanisms of tissue inflammation in autoimmune arthritis and may lead to the development of novel therapeutic modalities.

Disclosures
The authors have no financial conflicts of interest.

References


