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IL-15 and the Initiation of Cell Contact-Dependent Synovial Fibroblast-T Lymphocyte Cross-Talk in Rheumatoid Arthritis: Effect of Methotrexate

Maria-Eugenia Miranda-Carús, Alejandro Balsa, Marta Benito-Miguel, Carlos Pérez de Ayala, and Emilio Martín-Mola

To characterize the molecules responsible for synovial fibroblast-T lymphocyte (TL) cross-talk in rheumatoid arthritis (RA), synovial fibroblasts from patients with established RA (RASFibs) were cocultured with TLs from peripheral blood of early RA patients (RAPBTL). TLs from peripheral blood of healthy controls and from synovial fluid of RA served as controls. Adhesion molecules and cytokines were determined by flow cytometry, ELISA, and real-time PCR. RAPBTL (n = 20) induced an up-regulation of ICAM-1, intracellular IL-8, IL-6, IL-15, and surface IL-15 in cocultured RASFibs. In turn, RAPBTL showed an up-regulation of TNF-α, IFN-γ, IL-17, CD25, and CD69 expression. Responses seen with TLs from peripheral blood of healthy controls (n = 20) were significantly lower, whereas responses with TLs from synovial fluid of RA (n = 20) were maximal. Blocking Abs to IL-15 and CD54, but not an isotype-control Ab, down-regulated the increased TL cytokine and activation marker expression. Abs to CD69, CD11a, IL-17, TNF-α, and IFN-γ significantly decreased the up-regulation of RASFib cytokine and CD54 expression. Cocultures using 0.4-μm inserts did not result in up-regulation of surface molecules or cytokines. Methotrexate significantly inhibited RASFib/TL cross-talk signals and decreased adhesion of TL to RASFibs. In summary, RASFib production of IL-15 induces the proinflammatory cytokines TNF-α, IFN-γ, and IL-17 in cocultured TLs through a cell contact-dependent mechanism. In turn, these cytokines stimulate the expression of IL-15, IL-8, and IL-6 in RASFibs, thereby creating a feedback loop that favors persistent synovial inflammation. Methotrexate seems to disrupt this loop by decreasing cell adhesion. The Journal of Immunology, 2004, 173: 1463–1476.
TLs in the synovial membrane promote TNF-α secretion from synovial macrophages (23). Administration of soluble IL-15Rα prevents collagen-induced arthritis in mice and effectively reduces inflammation, synovial hyperplasia, and bone erosions (24). In addition, a phase I clinical trial in humans using a fully human anti-IL-15 mAb suggests that neutralization of IL-15 in patients with RA is effective and safe (25).

Our working hypothesis was that IL-15 on the surface of RASFib is able to modulate TL cytokine secretion through direct cell contact in the absence of local Ag recognition. These polyclonal IL-15-activated T cells would in turn stimulate synovial fibroblasts, thereby creating a juxtaocular loop favoring persistent inflammation. Our early arthritis clinic allowed the study of T cells from early RA patients who had not received disease-modifying antirheumatic drugs or steroids, thereby minimizing interference of drugs with in vitro TL responses.

Materials and Methods

Patients

Synovial membranes were obtained from 10 RA patients undergoing synovectomy or arthroplasty and from 10 osteoarthritis (OA) patients undergoing arthroplasty. Synovial fluid was obtained from 20 patients with established RA who were receiving treatment with oral methotrexate (MTX) and low-dose prednisone. Peripheral blood was obtained from 20 healthy controls and from 20 early RA patients benefiting at least four American College of Rheumatology criteria (26), who had never received disease-modifying drugs or corticosteroids and with a disease duration of <6 mo. La Paz University Hospital in Madrid, Spain, has a monochronic clinic that takes care of early arthritis patients referred from a wide primary care area. This facilitated recruitment of untreated early RA patients for the present study. Among early RA patients there were 7 male and 13 female, 15 (75%) tested positive for IgM rheumatoid factor, their ages were 18–80 years (mean 51.42, SD 18.53, median 50), duration of symptoms at first evaluation was from 2 to 26 wk (mean 11.53, SD 7.6, median 10), and disease activity score 28 (DAS28) (27) at first evaluation was from 4.71 to 7.49 (mean 5.98, SD 0.78, median 5.84). The study was approved by the Hospital Ethics Committee.

Culture of human fibroblasts

RASFibs and OASFibs were obtained by collagenase digestion (type I; Worthington Biochemical, Freehold, NJ) of human synovial tissue obtained at arthroplasty or synovectomy. Dermal fibroblasts were obtained by collagenase digestion of normal skin obtained from punch biopsies of five healthy volunteers. Cells were plated in 75-cm² flasks and grown in RPMI 1640 medium (In Vitrogen Life Technologies, Grand Island, NY) supplemented with 10% FCS (In Vitrogen Life Technologies), 2 mM l-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (In Vitrogen Life Technologies). Cells were passaged at dilution 1/2 when reaching 95% confluence, by gentle trypsinization (0.05% trypsin/0.53 mM EDTA; Invitrogen Life Technologies). Fibroblasts were used between passages 3 and 5. At this time, they appeared to be a homogeneous population of fibroblast-like cells that stained positive with anti-Thy-1 (CD90) Ab (28) and were negative for determining Ab present)/(cytokine/surface marker expression without neutralizing Ab present)

In vitro treatment with MTX

RASFibs were pretreated with MTX (1–500 nM; Sigma-Aldrich) 4 h before starting cocultures. Subsequently, TLs were added as described, without washing away MTX. In some experiments, to determine the contribution of adenosine release to the effect of MTX, adenosine deaminase (ADA; Type IV, calf intestinal, 0.125 U/ml; Sigma-Aldrich), the adenosine A2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMX; 10 µM; Sigma-Aldrich), or the adenosine A1 receptor antagonist 8-cyclopentyl-1-dipropylxanthine (DPCPX; 10 µM; Sigma-Aldrich) were added to the medium together with MTX. ADA was dialyzed against PBS overnight (4°C) before use, as described (31).

Percent inhibition by MTX was calculated as follows: 100 – [(cytokine/surface marker expression with neutralizing Ab present)/(cytokine/surface marker expression without neutralizing Ab present)] × 100.

Intrafascicular cytokine cross-talk in RA

Coculture conditions

Because in many cell systems, collagen and cytokine production vary with the stage of cell growth (29,30), experiments were performed with confluent fibroblast cultures prepared 24 h before contact. All experimental conditions were performed in duplicate and variation between replicates was <5%. Fibroblasts were seeded in six-well plates at 3 × 10⁵ cells/well. Twenty-four hours later, the separated TLs (2 × 10⁵ cells/well) were added, and cells were harvested after 6, 24, 48, 72, 96, and 120 h. TLs were collected by thorough washing with cold, serum-free medium. Subsequently, fibroblasts were detached with Trypsin-EDTA (Invitrogen Life Technologies). For detection of surface IL-15, fibroblasts were detached on ice with cold 5 mM EDTA in PBS.

In preliminary experiments, increasing TL numbers (T cells: 1 × 10⁵, 2 × 10⁵, 3 × 10⁵, 4 × 10⁵, and 5 × 10⁵ per well) were tested in coculture. A dose-response effect was observed, and 2 × 10⁵ TLs were used because an adequate amount of cytokine was induced to allow meaningful comparisons without using an excess of T cells. With TL numbers above 3 × 10⁵ per well, the observed TL responses were lower. This may be explained by a poor stochiometric proportion between TL number and fibroblast-derived cytokines.

Transwell system

A six-well Transwell system (Corning Costar, Cambridge, MA) was used to conduct some coculture experiments. The system consists of two compartments: a top well with a porous matrix (0.4 µm) and a bottom well. This setup allows coculture of two types of cells to grow in the same medium with soluble factors exchanged through the pores, while preventing direct contact between them. RASFibs were grown to confluence in the bottom well, and TLs were added either to the same well-allowing contact or in the top well-avoiding contact.

Paraformaldehyde (PFA) fixation of cells

Confluent fibroblasts in six-well plates were fixed on ice with chilled 2% PFA for 10 min. Subsequently, cells were thoroughly washed with chilled PBS to eliminate any traces of PFA before starting coculture.

Inhibition of cytokine and surface molecule up-regulation by neutralizing mAbs

Neutralizing mAbs against IL-15 (R&D Systems, Abingdon, U.K.), IL-2/IL-15Rβ (CD122), CD54 (R&D Systems), HLA class I (clone W6/32; Sigma-Aldrich, St. Louis, MO), or isotype control (BD Pharmingen) were incubated at 10 µg/ml with RASFibs for 30 min at 4°C. TLs were subsequently added without washing RASFibs. A neutralizing polyclonal goat IgG anti-IL-15Rα Ab (R&D Systems) or control goat IgG were used at 1 µg/ml. A recombinant human IL-15Rα Fc chimera (R&D Systems) or control human IgG were used at 100 ng/ml. Alternatively, neutralizing mAbs against CD11a/LFA-1, CD 69 (BD Pharmingen), TNF-α, IFN-γ, IL-17 (R&D Systems), or an isotype control mAb of irrelevant specificity (BD Pharmingen) were incubated at 10 µg/ml with TLs for 30 min at 4°C. TLs were then washed without addition to RASFibs. Percent inhibition was calculated as follows: 100 – [(cytokine/surface marker expression with neutralizing Ab present)/(cytokine/surface marker expression without neutralizing Ab present)] × 100.

TL adherence to synovial fibroblasts (heterotypic adherence)

The number of TLs that were firmly adhered to RASFibs was determined in 96-h cocultures, with and without MTX present. Adherent TLs were counted as described by Gadangi et al. (31): the nonadherent TLs were fixed with 4% PFA. The fixed cells were observed...
under an inverted microscope, and the number of adherent TLs was counted in three separate sites per well. All conditions were studied in triplicate. Percent inhibition of adhesion by MTX was calculated as follows: 100 – [(number of adhered TL with MTX present)/(number of adhered TL without MTX present)] × 100.

**In vivo effect of treatment**

Five patients with early RA donated blood for a second time, once disease activity had been controlled. Blood was also obtained from the five healthy subjects who had previously acted as controls for these patients. The patients were receiving oral MTX 15 mg weekly, except for one patient who was taking 25 mg per week. In addition, all five patients except for one were taking prednisone, 2.5 mg daily. IgM rheumatoid factor was positive at initial evaluation in four of these five patients. There were two male and three female; ages were 39.6 ± 13.83 (mean ± SD). DAS28 before initiation of treatment was 5.53 ± 0.76 (mean ± SD) and at the time of second blood drawing it was 1.95 ± 0.4. These patients were in remission as defined by a DAS28 score below 2.6 (32).

Cocultures of RAPBTL/RASFib and HCPBTL/RASFib were established. To minimize experimental variability, the same fibroblast lines were used for experiments with RAPBTL before and after treatment. Expression of cytokines, adhesion molecules, and activation markers in RAPBTL, HCPBTL, and RASFib were determined by flow cytometry in 96-h cocultures. Results were compared for each patient with results obtained before initiation of treatment. Percent inhibition was calculated as follows: 100 – [(cytokine/surface marker expression before starting treatment)/(cytokine/surface marker expression at disease control with MTX)] × 100.

**Intracellular cytokine staining, surface staining, and flow cytometry**

For intracellular cytokine staining, RASFibs and TLs were cocultured as described, and 10 μg/ml brefeldin A (Sigma-Aldrich) was added for the last 6 h. Importantly, no brefeldin A was added for detection of intracellular IL-15 as opposed to other intracellular cytokines. RASFibs and TLs were harvested, washed with PBS/2% FCS/0.01% NaN₃, permeabilized for 10 min with FACS permeabilizing solution 2 (BD Pharmingen), washed again, and incubated with various cytokine-specific mAbs or isotype-matched control mAbs conjugated to fluorochrome for 30 min at room temperature. After washing once with PBS/2% FCS/0.01% NaN₃ and once with PBS, cells were resuspended in 1% PFA and analyzed in a FACScan cytometry in 96-h cocultures. Results were compared for each healthy control with results obtained at initial experiment. For healthy controls, percent variation was calculated as follows: 100 – [(cytokine/surface marker expression at initial experiment)/(cytokine/surface marker expression at “disease control” experiment)] × 100.

**ELISAs**

RASFibs and TLs were cocultured under conditions identical with those described above, except that brefeldin A was omitted. Cell-free supernatants were collected and stored at −80°C. ELISAs for IL-15, IL-6, IL-8, TNF-α, IFN-γ, and IL-17 were performed using Duoset kits (R&D Systems), following the manufacturer’s instructions. Results were compared for each healthy control with results obtained at initial experiment. For healthy controls, percent variation was calculated as follows: 100 – [(cytokine/surface marker expression at initial experiment)/(cytokine/surface marker expression at “disease control” experiment)] × 100.

**Quantitative RT-PCR**

For RNA extraction, RASFibs and TLs were collected as described above. A population of TLs that remained attached to RASFibs despite extensive washing was detected. Treatment with trypsin detached RASFibs from the culture plates and at the same time was able to dissociate the remaining adherent TLs. For RASFib mRNA extraction, the resulting SFiBTL suspension was treated with a magnetically labeled anti-CD3 mAb (Miltenyi Biotec) and was passed through an Automacs (Miltenyi Biotec). Flow cytometric and mRNA analyses of those TLs that were strongly adherent to RASFibs demonstrated that there was no significant difference in cytokine or surface molecule expression with nonattached TLs, suggesting that direct contact between RASFibs and TLs is a dynamic process.

Total cellular RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) with in-column DNase treatment. For each sample, 1 μg of total RNA was subjected to reverse transcription using the Advantage RT for PCR kit (BD Clontech, Palo Alto, CA) according to the manufacturer’s instructions. Aliquots (1 μl) of the reverse transcription products were used for quantitative PCR in the LightCycler PCR and Detection System (Roche Molecular Biochemicals, Mannheim, Germany) using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics) as described by the manufacturer. The PCRs were set up in microcapillary tubes in a volume of 20 μl. The following sense and antisense primers were used: IL-6 sense, 5′-CAG CTA TGA ACT CCT TCT CCA CAA GC-3′; IL-6 antisense, 5′-CTG AGA TGC CTT CGA GGA TGT ACC G-3′ (product size, 201); IL-8 sense, 5′-ATG ACT TAC AAG CTT GCC GTG GTG-3′; IL-8 antisense, 5′-TCT CAG CTC TCT TCA AAT ACT TCT C-3′ (product size, 292); IL-15 sense, 5′-ATG AGA ATT TCG AAA CCA CAT-3′; IL-15 antisense, 5′-CTC CAA GAG AAG GCA GCT C-3′ (product size, 282); TNF-α sense, 5′-ATG AGC ACT AAG AGG AGT ATG CCT GCG-3′; TNF-α antisense, 5′-CTA CAA CTA GGG CTA CAG GCT TGT-3′ (product size, 286); IFN-γ sense, 5′-TGG AGC TCA TTC AGA TGT AG-3′; IFN-γ antisense, 5′-AGC CAT CAC TGG GAC TTG-3′ (product size, 307); IL-17 sense, 5′-TGG AGC TCA TAG TGA AGT GAG-3′; IL-17 antisense, 5′-GCC GAC ATG GTG GTG GAT-3′ (product size, 416); β-actin sense, 5′-GAG GCT GGA ATC GAC ACC GTC-3′; and β-actin antisense, 5′-GGA GGT AGT TTC GTG GAC GCC-3′ (product size, 225). Amplification of the PCR products was monitored by measuring SYBR Green I dye fluorescence. The transcript of human β-actin, amplified from the same cDNA samples, served as an external standard. Each sample was run in triplicate. Results were analyzed with LightCycler software version 3.5.3 (Roche Diagnostics). The identity of PCR products was confirmed by automated melting curve analysis after the amplification, which revealed the melting temperature (Tm) of the PCR products, followed by gel electrophoresis. In preliminary experiments, PCR products were further analyzed by direct sequencing (ABI PRISM310 Genetic Analyzer; PerkinElmer Applied Biosystems, Norwalk, CT). Quantities of specific mRNA in the sample were measured according to the corresponding gene-specific standard curve. The relative standard curve was prepared with duplicate serial dilutions of cDNA from a sample with the highest predicted levels of expression of the gene being investigated. The standards and the samples were simultaneously amplified using the same reaction mixture. The results were normalized as fold of induction: (cDNA from sample-treated cells/β-actin-treated cells)/cDNA sample untreated cells/β-actin untreated cells). The cDNA of untreated control cells normalized to the level of β-actin mRNA was ascribed a fold induction of 1. A nontemplate negative control (H₂O control) was run with every gene-specific primer. The no-RT-PCR control was run with only one primer pair that amplifies a sequence spanning an intron, to rule out contamination with genomic DNA (β-actin).

**Statistical analysis**

Comparison between groups was by Mann-Whitney U test. Paired samples were compared using a Wilcoxon matched pairs signed rank sum test. When appropriate, Bonferroni correction for multiple comparisons was applied.

**Results**

RAPBTLs activate cocultured RASFibs, as determined by upregulated cytokine and adhesion molecule expression

RASFibs constitutively expressed IL-15 on the cell surface, as determined by flow cytometry of nonpermeabilized cells (mean ± SD of positive cells was 9.2 ± 2.3% and MFI was 12.05 ± 1.8). Surface IL-15 expression progressively increased with time upon coculture with RAPBTLs (Fig. 1). At 96 h of coculture, percent positive cells ± SD was 24.4 ± 4.5 (p < 0.05 vs RASFibs alone) (Fig. 1) and MFI was 22.5 ± 2.6 (p < 0.05 vs RASFibs alone); at that time the stimulation index was 1.87 (p < 0.05). Constitutive expression of CD54 (ICAM-1) in resting RASFibs (60 ± 6.2% positive cells, MFI 38.44 ± 3.4) rapidly increased to a plateau at
24 h of coculture (Fig. 1). At 96 h of coculture, percent positive cells ± SD was 97.6 ± 1.2 (p < 0.05 vs RASFibs alone; Fig. 1) and MFI was 156.0 ± 6.5 (p < 0.05 vs RASFibs alone); at that time the stimulation index was 4.1 (p < 0.05).

In addition, RASFibs constitutively expressed intracellular IL-15, as determined by intracellular staining and flow cytometry (40.2 ± 2.4% positive cells; MFI 19.2 ± 2.0), but not IL-8 (0.8 ± 0.2% positive cells; MFI 0.3 ± 0.03) or IL-6 (0.6 ± 0.2%; MFI 0.40 ± 0.02) (Fig. 2). Intracellular IL-15 was detected without adding brefeldin A to the medium. Upon contact with RAPBTLs, a significant up-regulation of intracellular IL-15 was observed (87.5 ± 5.4% positive cells at 96 h of coculture, p < 0.05 vs RASFibs alone; MFI 31.4 ± 1.3, p < 0.05 vs RASFibs alone), together with an induction of IL-6 (31.4 ± 3.4% positive cells at 96 h, p < 0.05 vs RASFibs alone; MFI 16.4 ± 2.4, p < 0.05 vs RASFibs alone) and IL-8 (34.5 ± 4.3% positive cells at 96 h, p < 0.05 vs RASFibs alone; MFI 18.2 ± 1.9, p < 0.05 vs RASFibs alone) (Fig. 2). Augmented levels of IL-15 were observed without adding brefeldin A, whereas brefeldin A was necessary to detect intracellular IL-6 and IL-8. In parallel, real-time RT-PCR revealed an increased expression of IL-15, IL-6, and IL-8 mRNA in cocultured RASFibs (Fig. 2).

When compared with RAPBTLs, HCPBTLs were significantly less efficient at inducing RASFib activation (Figs. 1 and 2). In contrast, RASFTLs were significantly more efficient (Figs. 1 and 2) than RAPBTLs were.

IL-15 was not detected by ELISA in cell culture supernatants of resting or cocultured cells. In contrast, IL-6 and IL-8 concentrations in supernatants as determined by ELISA paralleled flow cytometry results. Specifically, IL-6 levels in 96-h culture supernatants of RASFibs alone, RASFibs cocultured with HCPBTLs, RASFibs cocultured with RAPBTLs, and RASFibs cocultured with RASFTLs were undetectable, 4.6 ± 0.5, 8.3 ± 0.8, and 15.5 ± 2.1 ng/ml, respectively (mean ± SD). IL-8 levels were undetectable, 3.5 ± 0.4, 7.2 ± 1.1, and 13.4 ± 2.2 ng/ml in culture supernatants of RASFibs alone, RASFibs cocultured with HCPBTLs, RASFibs cocultured with RAPBTLs, and RASFibs cocultured with RASFTLs, respectively.

Cocultures of TLs and synovial fibroblasts separated by 0.4-um inserts did not result in a significant increase of RASFib CD54 or cytokine expression (Figs. 1 and 2). This indicates that direct cell contact is mandatory to initiate cross-talk signals and that the role of soluble mediators released from resting cells is minor.

Resting RASFibs activate cocultured TLs, as determined by increased expression of CD69, CD25, IFN-γ, TNF-α, and IL-17

Because IL-15 shares many properties with IL-2 and has the capacity to activate TLs, we hypothesized that TLs would get stimulated in coculture with RASFibs and looked at TL activation markers and cytokine expression.
FIGURE 2. Resting RAPBTLs induce an up-regulation of IL-15, IL-8, and IL-6 protein and mRNA expression in cocultured RASFibs that is cell contact dependent. For comparison, shown are results using TLs from HCPBTLs and RASFTLs. A, Percent positive cells for intracellular IL-15, IL-8, and IL-6 staining by flow cytometry at 96-h coculture. Cocultures were established, allowing contact of RASFibs with TLs (open bars) and also using a 0.4 μM transwell system that separates both cell types into an upper and a lower chamber (filled bars). Each bar represents the arithmetic mean ± SD of 20 subjects per group. *, p < 0.05 vs resting RASFibs; †, p < 0.05 vs RASFibs cocultured with HCPBTLs; ‡, p < 0.05 vs RASFibs cocultured with RASFTLs (Mann-Whitney U test). B, Representative dot-plots of RAPBTLs, when freshly isolated (left panel) and after 96-h coculture with RASFibs (right panel). C, Real-time quantitative analysis of cytokine mRNA expression in RASFibs, at 48-h coculture with TLs. Shown is fold induction referred to expression in RASFibs that were not cocultured with TLs. Results for each cytokine are normalized to β-actin expression measured in parallel in each sample. Each bar represents the arithmetic mean ± SD of 20 subjects per group. *, p < 0.05 vs same population resting TLs; †, p < 0.05 vs cocultured HCPBTLs; ‡, p < 0.05 vs cocultured RAPBTLs (Mann-Whitney U test).

FIGURE 3. RASFibs, in a cell contact-dependent manner, up-regulate CD69 and CD25 expression in RAPBTLs. For comparison, shown are results using HCPBTLs and RASFTLs. A, Time-course diagrams of CD69 and CD25 expression on RAPBTLs cocultured with RASFibs, as determined by flow cytometry. Each point represents the arithmetic mean ± SD of 20 subjects per group. B, Representative dot-plots of RAPBTLs, when freshly isolated (left panel) and after 96-h coculture with RASFibs (right panel). C, Expression of CD69 and CD25 on RAPBTLs, HCPBTLs, or RASFTL, cocultured with RASFibs for 96 h. Cocultures were established allowing contact of RASFibs with TLs (open bars) and also using a 0.4 μM transwell system that separates both cell types into an upper and a lower chamber (filled bars). Each bar represents the arithmetic mean ± SD of 20 subjects per group. *, p < 0.05 vs same population resting TLs; †, p < 0.05 vs cocultured HCPBTLs; ‡, p < 0.05 vs cocultured RAPBTLs (Mann-Whitney U test).
CD69 expression was negligible in freshly isolated RAPBTLs (1.9 ± 0.5% positive cells, MFI 1.27 ± 0.4) and markedly increased with time in cocultures with RA SFibs (Fig. 3A); up-regulation was evident at 24 h and further augmented up to 72 h of coculture, reaching a plateau that was maintained up to 120 h (Fig. 3A). At 96 h, 37.3 ± 5.4% cells were CD69 positive (p < 0.05 vs resting RAPBTLs), with a MFI of 85.2 ± 5.4 (p < 0.05 vs resting RAPBTLs) (Fig. 3). CD25 expression was observed in 12.2 ± 1.9% of freshly isolated RAPBTLs with a MFI of 4.5 ± 0.7. An increase of CD25 expression could first be seen in 48-h cocultures and peaked at 96 h (Fig. 3A). At 96 h, 29.3 ± 4.7% cells were CD25 positive (p < 0.05 vs resting RAPBTLs), with a MFI of 17.4 ± 3.1 (p < 0.05 vs resting RAPBTLs) (Fig. 3).

Up-regulation of CD69 and CD25 was significantly pronounced in HCPBTLs cocultured with RASFibs (Fig. 3). RASFIBs demonstrated a high basal expression of CD69 and a moderately increased basal expression of CD25. When cocultured with RASFibs, RASFTLs demonstrated further up-regulation of CD69 and CD25 (Fig. 3C).

RABPBLs did not constitutively synthesize IFN-γ (0.08% ± 0.003 positive cells; MFI 0.02 ± 0.001), TNF-α (0.07 ± 0.002% positive cells; MFI 0.02 ± 0.001), or IL-27 protein (0.13 ± 0.03 positive cells; MFI 0.05 ± 0.002), as determined by intracellular staining and flow cytometry. In cocultures with RASFibs, intracellular IFN-γ, TNF-α, and IL-17 could be seen at 24 h, increased at 48 h, and peaked at 96 h, reaching a plateau that was maintained up to 120 h of coculture. At 96 h of coculture, intracellular TNF-α expression by flow cytometry (% positive cells ± SD) was 32.5 ± 5.3 (p < 0.05 vs resting RAPBTLs) with a MFI of 40.5 ± 3.8 (p < 0.05 vs resting RAPBTLs), IFN-γ expression was 27.3 ± 4.1% cells (p < 0.05 vs resting RAPBTLs) with a MFI of 17.36 ± 1.9 (p < 0.05 vs resting RAPBTLs), and IL-17 expression was 38 ± 5.2% cells (p < 0.05 vs resting RAPBTLs) with a MFI of 18.09 ± 2.5 (p < 0.05 vs resting RAPBTLs) (Fig. 4).

Again, RAPBTLs demonstrated heightened responses when compared with HCPBTLs and decreased responses when compared with RASFTLs (Fig. 4A), suggesting that a previously activated state facilitates responses to contact with RASFibs.

TNF-α levels in 96-h culture supernatants of RASFIBs alone, RASFibs cocultured with HCPBTLs, RASFibs cocultured with RAPBTLs, and RASFibs cocultured with RASFTLs were undetectable, 240 ± 20, 502 ± 53, and 1215 ± 152 pg/ml, respectively (mean ± SD), IFN-γ levels were undetectable, 180 ± 28, 386 ± 45, and 875 ± 92 pg/ml in culture supernatants of RASFIBs alone, RASFibs cocultured with HCPBTLs, RASFibs cocultured with RAPBTLs, and RASFibs cocultured with RASFTLs, respectively. IL-17 levels were undetectable, 120 ± 18, 338 ± 42, and 932 ± 220 pg/ml.

Real-time RT-PCR revealed an increased expression of TNF-α, IFN-γ, and IL-17 mRNA in TLs cocultured with RASFibs (Fig. 4C). Cocultures of TLs and synovial fibroblasts separated by 0.4-μm inserts did not result in a significant increase of TL activation marker or cytokine expression, suggesting that the role of soluble mediators released from resting cells is minor.

TL expression of ICAM-1 and LFA-1 was not modified in cocultures. CD40L and CTLA-4 were not expressed on resting TLs and were not induced up to 120 h after initiating cocultures.

**Role of IL-15 and CD54 on activation of TLs cocultured with RASFIBs**

We next sought to establish whether IL-15 expressed by RASFibs is implicated in activation of cocultured T lymphocytes. Up-regulation of CD69, CD25, IFN-γ, TNF-α, and IL-17 in TLs cocultured with RASFibs were significantly inhibited by a blocking anti-IL-15 mAb, by a blocking polyclonal goat IgG anti-IL-15Rα, and by a recombinant IL-15Rα/Fc chimera, but not by an irrelevant, isotype-matched mAb, not by normal goat IgG, and not by normal human IgG (Fig. 5A). A blocking anti-IL2-IL15-Rβ mAb was moderately effective at down-regulating TL activation (Fig. 5A). These data suggest that IL-15 plays a major role in cell contact-mediated activation of TLs by RASFibs.

Because direct cell contact seems to be mandatory for the initiation of TL/RASFib cross-talk, mAbs directed to components of the LFA-1/CD54 adhesion pathway were tested. A mAb to CD54 (ICAM-1) moderately decreased up-regulation of CD69, CD25, IFN-γ, TNF-α, and IL-17 (Fig. 5B). A combination of anti-CD54 with anti-IL15 Ab was synergistic at inhibiting TL activation (Fig. 5B). A blocking mAb anti-MHC class I did not modify TL activation induced by RASFibs (Fig. 5B).

**Role of TL surface molecules and cytokines on activation of RASFibs**

A blocking mAb to LFA-1 (CD11a) significantly decreased percent TL-induced activation of RASFibs, thereby confirming the importance of the CD54/LFA-1 adhesion pathway in this system (Fig. 5C). An Ab directed to CD69 was efficient at abrogating percent TL-induced up-regulation of RASFib cytokines and CD54 (Fig. 5C). TL cytokines that were induced in cocultures importantly contribute to activating RASFibs, as suggested by inhibition of RASFib stimulation when using mAbs to IL-17, TNF-α, or IFN-γ (Fig. 5C).

**Resting OASFibs and dermal fibroblasts do not express surface IL-15: behavior of OASFibs and dermal fibroblasts in coculture with TLs**

We were then interested in determining whether OASFibs and dermal fibroblasts constitutively express surface IL-15. As shown in Fig. 6A, no surface IL-15 expression could be detected on resting OASFibs or on dermal fibroblasts. In contrast, both OASFibs and dermal fibroblasts constitutively expressed intracellular IL-15 (Fig. 6A). Interestingly, intracellular IL-15 expression was significantly lower in resting OASFibs and dermal fibroblasts when compared with RASFibs (Fig. 6A).

We then tested the behavior of OASFibs and dermal fibroblasts in coculture with HCPBTLs, RAPBTLs, and RASFTLs. Cocultures of HCPBTLs with OASFibs or dermal fibroblasts resulted in minimal bidirectional activation, as determined by minimal elevation of fibroblast and T lymphocyte cytokines and activation markers (Fig. 6, A and B). This indicates that OASFibs and dermal fibroblasts do not have the capacity to induce an activated phenotype on HCPBTLs and that resting HCPBTLs do not have a significant effector function on OASFibs and dermal fibroblasts. Cocultures of RAPBTLs with OASFibs or dermal fibroblasts did result in bidirectional activation, although at significantly lower levels than those observed in cocultures of RAPBTLs with RASFibs (Fig. 6, A and B). This indicates that RAPBTLs are able to induce a low degree-activated state in OASFibs and dermal fibroblasts, which in turn can stimulate RAPBTLs, thereby establishing a proinflammatory loop. Cocultures of RASFTLs with OASFibs or dermal fibroblasts resulted in significant bidirectional activation, albeit at lower degrees than that observed in RASFib/RASFTL cocultures (Fig. 6, A and B). This indicates that activated RASFTLs are able to stimulate OASFibs and dermal fibroblasts and initiate an inflammatory process.

To evaluate the role of surface IL-15 on the differential behavior of RASFibs when compared with OASFibs and dermal fibroblasts, resting fibroblast cultures were fixed with PFA and then cocultured with RAPBTLs. PFA fixation of cells is known to prevent cytokine
FIGURE 4. RASFibs induce TNF-α, IFN-γ, and IL-17 protein and mRNA expression in cocultured freshly isolated RAPBTLs. This effect is cell contact dependent. For comparison, shown are results using HCPBTLs and RASFibs. A, TL cytokine expression at 96-h coculture with RASFibs, as determined by intracellular staining and flow cytometry. Cocultures were established allowing contact of RASFibs with TLs (open bars) and also using a 0.4 μm transwell system that separates both cell types into an upper and lower chamber (filled bars). Each bar represents the arithmetic mean ± SD of 20 subjects per group. *, p < 0.05 vs same population resting TLs; †, p < 0.05 vs cocultured HCPBTLs; ‡, p < 0.05 vs cocultured RAPBTLs (Mann-Whitney U test). B, Representative histograms demonstrate intracellular cytokine staining in RAPBTLs, cocultured with RASFibs for 96 h. Histograms show cytokine expression on resting (gray line) and cocultured (thick black line) TLs; the dotted black line represents staining with an isotype control mAb. C, Real-time quantitative analysis of cytokine mRNA expression in TLs, at 48-h coculture with RASFibs. Shown is fold induction referred to expression in resting TLs. Results for each cytokine are normalized to β-actin expression measured in parallel in each sample. Each bar represents the arithmetic mean ± SD of 20 subjects per group. *, p < 0.05 vs same population resting TLs; †, p < 0.05 vs cocultured HCPBTLs; ‡, p < 0.05 vs cocultured RAPBTLs (Mann-Whitney U test).

T cell subsets involved in interactions with RASFibs

We next examined whether the observed interactions are T cell subset-specific. To determine which subsets of T lymphocytes actively interact with RASFibs, CD4⁺, CD8⁺, CD45RO⁺, and CD45RA⁺ cells were magnetically isolated from peripheral blood of five early RA patients, and these subsets were tested in coculture experiments. Although no significant differences were observed between CD4⁺ and CD8⁺ subsets, CD45RO T cells demonstrated a higher capacity to stimulate and be stimulated by RASFibs when compared with CD45RA TLs (Fig. 7). Observed differences persisted when applying Bonferroni correction for multiple comparisons.

Because RAPBTLs demonstrated a higher capacity to activate and be activated by RASFibs, RAPBTLs were carefully examined for the presence of classical activation markers such as CD69, CD25, HLA-DR, and CD40L. Interestingly, early RA patients did not demonstrate an increased expression of CD69, CD25, or CD40L when compared with healthy controls. As reflected in Fig. 3C, expression levels of CD25 and CD69 were similar in RAPBTLs and HCPBTLs. In addition, no expression of CD40L could be detected in RAPBTLs or HCPBTLs. RAPBTLs demonstrated an increased expression of HLA-DR (mean ± SD, 7.1 ± 3.9; median 5.5; range 2–14) when compared with HCPBTLs (4.2 ± 2.5; median 4.0; range 1–9), but this difference was small and not relevant. Results of coculture experiments were compared between seven early RA patients whose HLA-DR expression was above the maximum observed in HCPBTLs and 13 RA patients whose HLA-DR expression was below the maximum observed in HCPBTLs. The early RA group with higher HLA-DR expression demonstrated a slightly higher capacity to activate and be activated by RASFibs when compared with the early RA group with lower HLA-DR expression, although differences were not significant (Fig. 7).

Excluding an allogeneic effect

Previous work of others has demonstrated that no allogeneic effects are observed in cocultures of human fibroblasts with allogeneic T cells (8, 34). Nevertheless, it was important to exclude subtle allogeneic effects in our system. To this end, RASFibs were cocultured with autologous or allogeneic RAPBTLs within a single experiment (Fig. 8). Results from three such experiments...
shown comparable percent activation when autologous or allogeneic T lymphocytes were cocultured with RASFibs (Fig. 8).

**In vitro effect of MTX on RASFib/TL interactions**

Because MTX is still the most commonly used drug in RA and because most of our patients require MTX as a disease modifying drug immediately after diagnosis is confirmed, the in vitro effect of MTX on RASFib/TL interactions was tested. A dose-dependent inhibition of RASFib/TL cross-talk signals was observed. As an example, shown are dose-response curves of RASFib surface IL-15 and of RAPBTL CD69 expression in 96-h cocultures (Fig. 9, A and B). Low-percent inhibition was already seen with MTX 1 nM, and the effect was maximal with MTX 100 nM (Fig. 9, A and B).

As seen in Fig. 9C, CD54 up-regulation in RASFibs cocultured with RAPBTLs was not inhibited by MTX 100 nM. In contrast, MTX (100 nM) induced a significant inhibition of IL-15, IL-6, and IL-8 up-regulation in RASFibs cocultured with RAPBTLs for 96 h. Likewise, up-regulation of CD69, CD25, IFN-γ, and IL-17 in RAPBTLs cocultured with RASFibs for 96 h was significantly inhibited by MTX 100 nM (Fig. 9D). In contrast, CD11a-LFA1 expression on RAPBTLs was not modified by MTX (Fig. 9D). The effect of MTX on RASFib/TL cross-talk signals was reversed by ADA (0.125 IU/ml), suggesting that adenosine release mediates inhibition of RASFib/TL cross-talk (Fig. 9, A–D). The effect of MTX was also reversed by the adenosine A2 receptor antagonist DMPX (10 μM; Fig. 9), but not by the chemically related A1 receptor antagonist DPCPX (10 μM; data not shown); this suggests that adenosine released by MTX acts through A2 receptors. Treatment with MTX did not result in decreased cell viability as determined by propidium iodide and annexin V staining.

Because MTX has been described to decrease adhesion of neutrophils to endothelium and to dorsal fibroblasts (35), we sought to determine whether it interferes with adhesion of TLs to RASFibs. The number of RAPBTLs that were firmly adhered to fibroblasts was counted in MTX-treated and untreated 96-h cocultures. Interestingly, a marked decrease in the number of adherent lymphocytes was seen in the MTX-treated wells (Fig. 9E), suggesting that the effect of MTX on RASFib/TL cross-talk signals may be mediated, at least in part, by decreased cell adhesion. The effect of MTX on adhesion was dose-dependent: it was reversed by ADA (0.125 IU/ml) and by DMPX (10 μM; Fig. 9E), but not by DPCPX (data not shown).

**In vivo effect of treatment**

We also sought to determine whether disease control with treatment results in decreased TL–RASFib cross-talk signals. Five patients with early RA donated blood for a second time, once disease activity had been controlled. Blood was also obtained from the five healthy subjects who had previously acted as controls for these patients. As seen in Fig. 10, bidirectional activation in RASFib/TL cocultures was significantly decreased when compared with responses before initiation of treatment. Experimental variation observed in healthy controls was minimal. These results indicate that the basal activated state displayed by PBTLs of early RA patients is down-regulated in vivo by disease-modifying agents while controlling disease activity.

**Discussion**

The results presented herein suggest that RASFibs, by interacting with TLs, can be important contributors to the initiation and perpetuation of inflammation in RA. RASFibs constitutively express intracellular and surface IL-15. Constitutively expressed IL-15 is

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**FIGURE 5.** Molecules implicated in TL/RASFib cross-talk. RASFibs were cocultured with RAPBTLs in the presence of specific neutralizing Abs. Surface or intracellular staining and flow cytometry was done in 96-h cocultures. Percentage inhibition by neutralizing Abs is shown, referred to cytokine or surface molecule expression in cocultures without neutralizing Abs. Percent inhibition was calculated as follows: 100 − (cytokine/surface marker expression with neutralizing Ab present)/cytokine/surface marker expression without neutralizing Ab present] × 100. A. Activation of RAPBTLs induced by cocultured RASFibs is markedly decreased in the presence of a neutralizing anti-IL-15 mAb, a neutralizing goat polyclonal anti-IL-15, a chimeric IL-15α-Fc construct, and a neutralizing anti-IL-2Rβ mAb, but not when a nonspecific isotype control mAb, goat IgG, or human IgG is added to the culture. B. Activation of RAPBTLs induced by cocultured RASFibs is markedly decreased in the presence of blocking mAbs to IL-15 or CD54, but not when an anti-IL-1A class I or an irrelevant, isotype control mAb is added to the cultures. C. RASFib activation induced by RAPBTLs is markedly decreased when using mAbs to CD69, LFA-1 (CD11a), TNF-α, IFN-γ, or IL-17, but not an isotype control mAb. Each bar represents the arithmetic mean ± SD of 20 subjects per group, except for experiments using anti-IL-15α, IL-15α-RαFc, and anti-IL-2Rβ, in which only 12 subjects per group were tested. *, p < 0.01 vs isotype control or normal IgG.
biologically active on TLs and in turn gets up-regulated upon contact with T cells. This constitutes a feedback loop that favors the persistence of the inflammatory process in RA. Interestingly, RASFibs share constitutive surface expression of IL-15 with human spleen-derived fibroblasts (19), whereas OASFibs and fibroblasts from other locations do not demonstrate surface IL-15 unless stimulated by inflammatory cytokines (20) or derived from tumor stroma (36). In contrast, fibroblasts from all sources do constitutively express intracellular IL-15 (15).

When compared with PBTLs of healthy controls, PBTLs from early RA patients were more efficient at inducing activation of RASFibs and also were more easily activated in cocultures, indicating the existence of a previously activated state favoring cell contact-mediated responses. However, expression of CD25, CD69, and CD40L was not elevated in RAPBTLs, and expression of HLA-DR was only slightly increased when compared with HCPBTLs. Further studies are needed to elucidate the mechanisms underlying the functional behavior of RAPBTLs in coculture with RASFibs. Functional differences between peripheral blood T cells of RA and healthy controls have previously been described, and the mechanisms responsible for these differences are largely unknown (37). In our system, the observed preactivated state was no longer present in PBTLs of early RA patients once disease activity was controlled with treatment (MTX + low-dose prednisone). Freshly isolated TLs from RA synovial fluid, with increased basal CD69 and CD25 expression, were more efficient at RASFib activation when compared with TLs from peripheral blood of early RA. This confirms the in vivo relevance of the pathways detected in vitro.

Experiments with transwell inserts suggest that direct cell contact is mandatory to initiate RASFib-TL cross-talk. Once intercellular cross-talk is initiated by direct contact, soluble factors such as IL-17, IL-6, and IL-8 are liberated, which contribute to augmenting the proinflammatory loop.

The observed effects are not attributable to an allogeneic reaction because a blocking anti-MHC class I mAb did not display any significant effect. In addition, comparable percent activation was observed in autologous and allogeneic RASFib/RAPBTL cocultures. Previous work of others has shown that cocultures of fibroblasts and T lymphocytes from different donors do not result in allogeneic reactions, and this is attributable to poor or absent fibroblast expression of costimulatory molecules CD80 and CD86 (8, 38). In fact, Corrigall et al. (38) reported that lack of CD80 and

FIGURE 6. Comparison of RASFib, OASFib, and dermal fibroblast behavior in 96-h coculture with TLs. A, Surface and intracellular IL-15 expression in RASFibs, OASFibs, and dermal fibroblasts, under resting conditions and at 96 h. coculture with HCPBTL, RAPBTL or RASFTL. *p < 0.005 vs RASFib; †p < 0.05 vs resting fibroblasts of same tissue origin. Each bar represents the arithmetic mean ± SD of 20 subjects per group (coculture with RASFibs) or 10 subjects per group (cocultures with OASFibs and dermal fibroblasts). B, Expression of CD69 and intracellular IL-17 in HCPBTLs, RAPBTLs, and RASFTLs, under resting conditions and in coculture with RASFibs, OASFibs, or dermal fibroblasts. *, p < 0.05 vs TLs cocultured with RASFibs; †, p < 0.005 vs resting TLs of same origin. Each bar represents the arithmetic mean ± SD of 20 subjects per group (coculture with RASFibs) or 10 subjects per group (cocultures with OASFibs and dermal fibroblasts). C, Expression of CD69 and intracellular IL-17 in RAPBTLs cocultured with PFA-fixed RASFibs, OASFibs, or dermal fibroblasts. Cells were cocultured in medium alone, with a neutralizing anti-IL-15 mAb, or with an isotype control mAb. *, p < 0.01 vs resting RAPBTLs. Each bar represents the arithmetic mean ± SD of 10 subjects per group.
CD86 on synovial fibroblasts results in T cell anergy with no response to allogeneic stimulation and a conserved response to recombiant human IL-2.

Our initial hypothesis stated that surface IL-15 is key to contact-dependent activation of TL. This was confirmed in experiments with neutralizing Abs to IL-15, IL-15Rα, and IL-8β and with a soluble IL15Rα-Fc chimera. IL-17, IFN-γ, and TNF-α expression in cocultured TLs were significantly decreased by blockade of IL-15 and IL-15Rα, suggesting that IL-15 plays a major role in TL cytokine induction (Fig. 11). Likewise, increased CD69 and CD25 expression were IL-15 dependent (Fig. 11).

No secretion of IL-15 could be detected in supernatants of resting RASFibs or of RASFibs cocultured with T lymphocytes. This has been extensively documented in previous reports. Although IL-15 is constitutively expressed by fibroblasts of various localizations, secretion to the extracellular space has rarely been demonstrated (19, 20, 39).

The secretion of IL-15 is very exquisitely regulated and not yet well understood (39). IL-15Rα has a very high affinity for IL-2 (Kd = 10 nM) compared with the low affinity of IL-2Rα for IL-2 (Kd = 10 pM) (39). It has been proposed that the high affinity of IL-15Rα results in an extremely rapid uptake of secreted IL-15 by producer cells and also by neighboring cells, thereby preventing detection of IL-15 in culture supernatants (40, 41) (Fig. 11). This mechanism implies autocrine as well as paracrine activation and has been termed “intercellular recirculation of IL-15” (40). Furthermore, Dubois et al. (41) demonstrated that most of the IL-15 detected on cell surfaces is bound to IL-15Rα (Fig. 11) and that the complex IL15/IL-15Rα is biologically active on neighboring cells through direct cell contact (41): in fact, IL-15Rα is able to present IL-15 in trans to cells expressing the IL-15Rβ and IL-15Rγ chains (41) (Fig. 11). Surface, IL-15Rα-bound IL-15 has a wider biological activity than does soluble IL-15 because the signal induced in trans by the IL-15/IL-15Rα complex can stimulate efficiently at
picomolar concentrations the proliferation of both βγ- and IL-15Rα-bearing cells in a similar manner (41). That is, the presence of surface IL-15Rα-bound IL-15 is synonymous with active IL-15 secretion (Fig. 11), and the level of expression of surface IL-15 in a fibroblast cell population may reflect the rate of internalization of the IL-15/IL-15Rα complex. It has been proposed on RAPBTLs, in a dose-dependent manner. This effect is reversed by ADA (0.125 IU/ml) and by the adenosine A2 receptor antagonist DMPX (10 μM). C and D, MTX (100 nM) inhibits up-regulation of RASFib (C) and RAPBTL (D) surface molecule and intracellular cytokine expression. Inhibition is reversed by ADA and by DMPX. E, MTX decreases adhesion of TLs to RASFib in a dose-dependent manner; the effect is reversed by ADA and by DMPX. Each bar represents the mean ± SD of 20 patients. *, p < 0.01 vs expression in conditions without MTX present.

FIGURE 9. Methotrexate down-regulates bidirectional RASFib/TL activation. RASFibs were pretreated with MTX (10–500 nM) 4 h before starting cocultures. Subsequently, RAPBTLs were added to the culture, without washing away MTX. Surface or intracellular staining and flow cytometry was done at 96-h coculture. Percentage inhibition by MTX is shown, referred to cytokine or surface molecule expression in cocultures without MTX. Percent inhibition was calculated as follows: 100 − [(cytokine/surface marker expression with MTX present)/cytokine/surface marker expression without MTX] × 100. A and B, MTX inhibits coculture-induced up-regulation of surface IL-15 on RASFibs and of CD69.

FIGURE 10. Control of disease with treatment results in down-regulation of TL/RASFib cross-talk signals. PBTLs were isolated for a second time from five patients with early RA, once disease control had been achieved with oral MTX plus low-dose prednisone. Blood was also obtained from the five healthy subjects who had previously served as controls for these patients. Cocultures with RASFibs were established (RASFibs were the same fibroblast lines used for experiments before treatment). Expression of cytokines, adhesion molecules, and activation markers in TLs and RASFibs was determined by flow cytometry in 96-h cocultures. Results were compared for each patient with results obtained before initiation of treatment. Percent inhibition was calculated as follows for each patient: 100 − [(cytokine/surface marker expression before starting oral MTX)/cytokine/surface marker expression at disease control with MTX] × 100. For healthy controls, percent variation was calculated as follows: 100 − [(cytokine/surface marker expression at initial experiment)/(cytokine/surface marker expression at “disease control” experiment)] × 100. Each bar represents the mean ± SD of five subjects. *, p < 0.01 vs expression before starting MTX.

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that IL-15 may also interact with the plasma membrane independent of its receptor (Fig. 11). Potentially, this could occur indirectly through interactions with as yet unidentified receptors or directly through either one of the IL-15 abnormal N terminus signaling peptides or by the addition of a predicted C terminus GPI anchor (42). In summary, IL-15 is not usually secreted to the extracellular space of RASFibs constitutively express surface IL-15. Most cell surface IL-15 seems to be attached to IL-15Rα, which rapidly takes up secreted IL-15. Alternatively, surface IL-15 may be directly anchored to the plasma membrane through a signal peptide or GPI or indirectly bound to as yet unknown receptors (these three alternative mechanisms have been depicted as “X”). B, IL-15 bound to IL-15Rα on the surface of RASFib is able to stimulate neighboring T cells in trans, through IL-2Rβ and common γ-chains. In addition, secreted IL-15 and IL-15 anchored to plasma membrane may stimulate adherent T cells in cis by binding T cell IL-15Rα. T cell IL-15Rα would compete with fibroblast IL-15Rα for secreted IL-15. C, Fibroblast IL-15 up-regulates T cell CD69, CD25, IL-17, TNF-α, and IFN-γ expression that contribute to further increasing IL-15 and induce fibroblast IL-6 and IL-8 secretion. This constitutes a feedback loop that perpetuates the inflammatory process.

FIGURE 11. Fibroblast IL-15 stimulates T cells through direct cell contact. A, RASFibs constitutively express surface IL-15. Most cell surface IL-15 seems to be attached to IL-15Rα. This mechanism is favored by the high affinity of IL-15Rα, which rapidly takes up secreted IL-15. Alternatively, surface IL-15 may be directly anchored to the plasma membrane through a signal peptide or GPI or indirectly bound to as yet unknown receptors (these three alternative mechanisms have been depicted as “X”). B, IL-15 bound to IL-15Rα on the surface of RASFib is able to stimulate neighboring T cells in trans, through IL-2Rβ and common γ-chains. In addition, secreted IL-15 and IL-15 anchored to plasma membrane may stimulate adherent T cells in cis by binding T cell IL-15Rα. T cell IL-15Rα would compete with fibroblast IL-15Rα for secreted IL-15. C, Fibroblast IL-15 up-regulates T cell CD69, CD25, IL-17, TNF-α, and IFN-γ expression that contribute to further increasing IL-15 and induce fibroblast IL-6 and IL-8 secretion. This constitutes a feedback loop that perpetuates the inflammatory process.

activation of peripheral blood TLs (43) and leukocytes (44). It remains to be determined whether the effect of CD54 is exclusively related to adhesion or whether CD54 has an intrinsic activating effect (45, 46). Simultaneous blockade of IL-15 and CD54 was synergistic at decreasing TL-RASFib cross-talk. Blocking mAbs to CD11a likewise decreased RASFib activation induced by cocultured TLs, thereby suggesting a role for β2 integrins in this coculture system and stressing the importance of cell adhesion in RATL-SFib cross-talk.

The TL surface marker CD69 is important in contact-dependent RASFib activation, because a mAb to CD69 moderately decreased the up-regulated RASFib expression of IL-15, IL-6, IL-8, and CD54 induced by cocultured TLs. CD69 has been implicated in contact-mediated TL activation in previous in vitro studies (23, 47). In contrast, recent work (48) with CD69 knockout mice suggests that CD69 may have an anti-inflammatory role mediated by increased production of TGF-β, leading to decreased proinflammatory cytokine production. Further studies are needed to clarify discrepancies in the putative function of CD69 derived from in vitro studies as opposed to in vivo animal models.

The induction of IL-17 in cocultures is an important contributor to the feedback loop. A blocking mAb to IL-17 significantly decreased contact-dependent RASFib IL-6 and IL-8 expression and had a moderate inhibitory effect on up-regulation of CD54 and IL-15. TL-derived TNF-α and IFN-γ are also important players, because RASFib activation was down-regulated by specific blocking mAbs. IL-17 is a novel cytokine produced by activated T cells (49, 50), and elevated levels of IL-17 have been described in synovial fluids of RA but not OA patients (49, 50). In addition, CD4CD45RO T cells in synovial tissue of RA patients are immunoreactive with anti-IL-17 Abs (50), and cultured pieces of RA synovium produce IL-17 (51). This, together with the findings in our coculture system, suggests that blocking the action of IL-17 may help control disease activity in RA.

In an excellent previous study, Yamamura et al. (8) reported that TLs from healthy donors and RA patients do not show any sign of activation when cocultured with RASFibs; however, RASFibs were stimulated upon contact with T cells, as determined by induction of IL-6, IL-8, and PGE2 production (8). This contrasts with our findings and with recent evidence that PBTLs proliferate when cocultured with RA synovial fibroblasts (12). We can only speculate that factors such as lower coculture time frame in Yamamura’s system, previous treatment of patients, and/or a higher T cell: RASFib ratio may explain differences.

MTX attenuated the RAPBTL activation induced by RASFibs in vitro and it also modulated the up-regulation of RASFib IL-15, IL-6, and IL-8 induced by RAPBTLs. At the same time, MTX significantly decreased RAPBTL adhesion to RASFibs. Because cell contact is important for the bidirectional SFib/TL cross-talk, decreased SFib/TL adhesion could be one mediator of MTX effect in this coculture system. MTX is the most commonly used drug in RA and its mechanism of action is still being investigated (52). At the low doses used for RA treatment, MTX induces release of adenosine to the extracellular space (52). Adenosine is a potent anti-inflammatory autacoid and mediates MTX inhibition of neutrophil adhesion to endothelium and fibroblasts (52). In our system, the effect of MTX was reversed by ADA and by the adenosine A2 receptor antagonist DMPX. This suggests that adenosine release plays a role in the observed effects acting through A2 receptors, similar to the effect of adenosine on neutrophil adhesion to endothelial cells (53). MTX did not modify the expression of CD54 or CD11a in resting or cocultured cells. This may indicate that MTX effect on adhesion is mediated by changes in the affinity
and/or adversity of adhesion molecules, consistent with previous reports that LFA-1 function can be modulated without changing cell surface expression levels (54). Our experiments with PBTs of early RA patients who subsequently received oral MTX showed that MTX, while controlling disease activity, modifies the capacity of peripheral blood T cells to activate and be activated by RASFs. Previous reports have demonstrated in vivo effects of MTX in animal models, such as decreased recruitment of TLs to the arthritis joint of mice with collagen-induced arthritis (55). Clinical improvement with MTX in rheumatoid synovitis has been associated with a significant decrease in the number of T cells infiltrating the synovial membrane (56, 57) and also with decreased migration and articular recruitment of neutrophils (58).

In summary, constitutively expressed IL-15 on RA synovial fibroblasts is able to stimulate freshly isolated T cells. This generates a positive feedback loop whereby IL-15 induces and maintains T cell synthesis of TNF-α, IFN-γ, and IL-17, which can further activate RASFs to produce IL-15 and also IL-6 and IL-8 (Fig. 11). TLs from early RA patients particularly contribute to this effect, showing increased responses when compared with healthy controls. This implies that circulating T cells from RA patients may participate in the initiation and perpetuation of the RA joint inflammation. The T cell/synovial fibroblast cross-talk loop could favor residual inflammation in RA patients who fail to respond to TNF-α treatment. MTX, still the most commonly used drug in RA, interferes with RASF/TL cross-talk by decreasing heterotypic adhesion.

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