

# Expression of Interleukin 12 Receptor (IL-12R) and IL-18R on CD4+ T Cells from Patients with Rheumatoid Arthritis

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**ABSTRACT. Objective.** Interleukin 12 (IL-12) and IL-18 synergistically induce interferon- $\gamma$  (IFN- $\gamma$ ) production by T cell infiltrates in rheumatoid arthritis (RA). To investigate this synergism, we examined the expression and regulation of IL-12 receptor (IL-12R) and IL-18R on peripheral blood (PB) and synovial tissue (ST) CD4+ T cells from patients with RA.

**Methods.** The mRNA and cell surface expression of IL-12R and IL-18R in CD4+ T cells were determined by reverse transcriptase-polymerase chain reaction and flow cytometry, respectively. IFN- $\gamma$  and IL-4 production by CD4+ T cells stimulated with phorbol myristate acetate (PMA) and calcium ionophore A23187 was measured by intracellular cytokine staining and flow cytometry.

**Results.** Despite the negligible expression of IL-12R on fresh cells, PB CD4+ T cells from RA patients expressed higher levels of both IL-12R $\beta$ 1 and  $\beta$ 2 subunits after stimulation with anti-CD3 antibody (Ab) than the cells of healthy controls. ST CD4+ T cells contained mRNA transcripts encoding IL-12R $\beta$ 1 and  $\beta$ 2, and expressed detectable levels of these 2 subunits on the cell surface. Their IL-12R expression was markedly augmented by costimulation with anti-CD3 Ab and IL-18. In contrast, IL-18R $\alpha$  was expressed on freshly isolated PB CD4+ T cells from RA patients and controls, and the level of expression was higher in RA. IL-18R $\alpha$ + CD4+ T cells were further increased in the ST lesion, where IL-18R $\beta$  mRNA was constitutively detected. IL-12R $\beta$ 1 and  $\beta$ 2 were induced mainly on IL-18R $\alpha$ + CD4+ T cells after anti-CD3 Ab stimulation. PMA and A23187-activated ST CD4+ T cells mostly expressed IL-18R $\alpha$  and produced high levels of IFN- $\gamma$ .

**Conclusion.** These results indicate that IL-18R-expressing CD4+ T cells are accumulated in the ST of patients with RA, where the functional IL-12R is locally induced by stimuli such as CD3 activation and IL-18. Activation of both cytokine receptors may be necessary for the IFN- $\gamma$ -dominant cytokine response. (J Rheumatol 2004;31:448–56)

## Key Indexing Terms:

CD4+ T CELLS      INTERLEUKIN 12 RECEPTOR      INTERLEUKIN 18 RECEPTOR  
RHEUMATOID ARTHRITIS      Th1 CELLS

The chronically inflamed synovial tissue (ST) of rheumatoid arthritis (RA) is highly infiltrated with T cells, primarily mature memory CD45RO+ CD4+ T cells. The majority of CD4+ T cells are of the Th1 phenotype, characterized by their ability to preferentially produce interferon- $\gamma$  (IFN- $\gamma$ )<sup>1</sup>. Although the role of T cells in the pathogenesis remains controversial, recent evidence has suggested that the Th1 cytokines IFN- $\gamma$  and interleukin 17 (IL-17), together with cell surface molecules, activate synovial macrophages,

fibroblast-like cells, and osteoclasts, contributing to both the chronic inflammation and joint destruction<sup>1,2</sup>. The local synthesis of IFN- $\gamma$  in RA joints may be induced primarily by the synergistic effect of IL-12 and IL-18 produced by activated macrophages and dendritic cells<sup>3–5</sup>. We have demonstrated that IL-12, a critical cytokine required for the development of Th1 responses, is present at low concentrations in the ST, but an abundance of IL-18 augments IL-12 responsiveness of T cells<sup>3,5</sup>.

The ability of IL-12 to activate T cells is mediated by the functional high-affinity IL-12 receptor (IL-12R), a heterodimer of IL-12R $\beta$ 1 and  $\beta$ 2 subunits<sup>6,7</sup>. The IL-12R $\beta$ 2 subunit functions as the primary signal-transducing component that activates the central transcription factor for IFN- $\gamma$  synthesis and Th1 cell differentiation, signal transducer and activator of transcription-4 (STAT4). IL-12R $\beta$ 1 is expressed on both Th1 and Th2 cells, but IL-12R $\beta$ 2 is persistently expressed on Th1 cells but not on Th2 cells. IL-12R $\beta$ 2 expression has been reported to be elevated in Th1 cell-

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mediated pathological conditions, such as tuberculosis<sup>8,9</sup>, tuberculoid leprosy<sup>10</sup>, sarcoidosis<sup>9</sup>, hypersensitivity pneumonitis<sup>11</sup>, Crohn's disease<sup>12</sup>, and multiple sclerosis<sup>13</sup>, but not in allergic asthma, a Th2 cell-driven disease<sup>9</sup>.

The functional IL-18R is structurally homologous to the IL-1R system, and is thus composed of 2 subunits, IL-1R-related protein (IL-1Rrp; IL-18R $\alpha$ ) and IL-1R accessory protein-like protein (AcPL; IL-18R $\beta$ )<sup>14</sup>. The IL-18R $\alpha$  subunit transduces a signal that induces activation of the transcription factors nuclear factor  $\kappa$ B (NF- $\kappa$ B) and activation protein 1 (AP-1). High levels of the inducible IL-18R $\alpha$  are selectively expressed on the cell surface of Th1 cells but not Th2 cells<sup>15,16</sup>. IL-18R $\alpha$  has been detected on lymphocytes and macrophages in the ST of patients with RA<sup>4</sup> and in the pulmonary lesion of sarcoidosis<sup>17</sup>.

To elucidate the mechanism of the synergism between IL-12 and IL-18 in perpetuating Th1 cytokine predominance in RA joints, we investigated the regulation of IL-12R and IL-18R expression on CD4+ T cells in patients with RA.

## MATERIALS AND METHODS

**Patients and samples.** Patients with RA were diagnosed according to the revised 1987 criteria of the American College of Rheumatology (formerly, the American Rheumatism Association)<sup>18</sup>. Most patients were receiving prednisolone at  $\leq 5$  mg/day and various disease-modifying antirheumatic drugs. ST samples were obtained from RA patients at the time of surgical treatment. The mean age of the patients was  $63.3 \pm 10.4$  years (mean  $\pm$  SD, range 42–78 years,  $n = 38$ ). Their laboratory data were as follows; erythrocyte sedimentation rate  $53 \pm 21$  mm/h, C-reactive protein  $36 \pm 44$  mg/l, and IgM class rheumatoid factor  $155 \pm 170$  U/ml. Peripheral blood (PB) samples were also collected from 18 age-matched healthy volunteer controls. All patients and controls gave informed consent.

**Isolation of CD4+ T cells.** PB mononuclear cells (PBMC) were prepared from heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. ST mononuclear cells were prepared as described<sup>19</sup>. Briefly, fresh ST samples were fragmented and digested with collagenase and DNase for 1 h at 37°C. After removing tissue debris, cells were washed well with RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA). PB and ST cells were resuspended in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FCS; Life Technologies). Lymphocytes were enriched by removing adherent cells after a 45-min incubation of the cells at 37°C in 6-well plates (Costar, Cambridge, MA, USA). CD4+ T cells were purified from lymphocytes by positive selection using anti-CD4 monoclonal Ab (mAb)-coated magnetic beads (Dynabeads M-450 CD4; DYNAL, Oslo, Norway) and detaching solution (DetachaBead CD4/CD8; DYNAL), according to the manufacturer's instructions.

CD4+ T cells were resuspended in complete medium: RPMI 1640 medium supplemented with 5% heat-inactivated human AB serum, 25 mM HEPES (Life Technologies), 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin.

**Induction of IL-12R $\beta$ 1 and  $\beta$ 2.** CD4+ T cells were dispensed into the wells of 24-well microtiter plates (Costar), which were coated with 100 ng/ml anti-CD3 mAb (Immunotech, Marseille, France)<sup>20</sup>, at a density of  $2 \times 10^5$  cells/ml. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were harvested 48 h later and the expression of IL-12R $\beta$ 1 and  $\beta$ 2 was determined by flow cytometry, because the kinetics of IL-12R induction indicated that levels of IL-12R $\beta$ 1 and  $\beta$ 2 expression reached a maximum mostly at 48 h stimulation.

**Flow cytometry.** For determination of the cell surface expression of IL-12R $\beta$ 1 and  $\beta$ 2 and IL-18R $\alpha$ , PBMC and CD4+ T cells were stained with rat

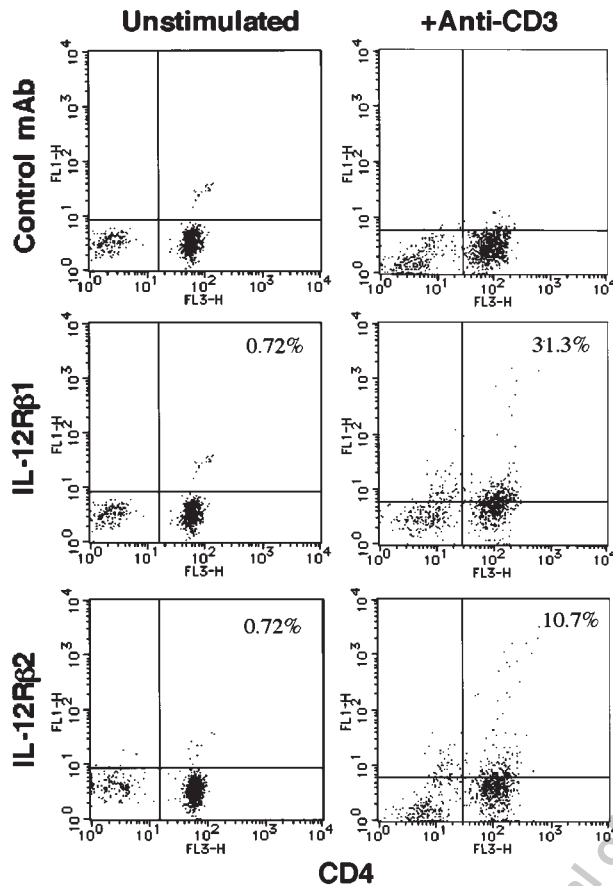
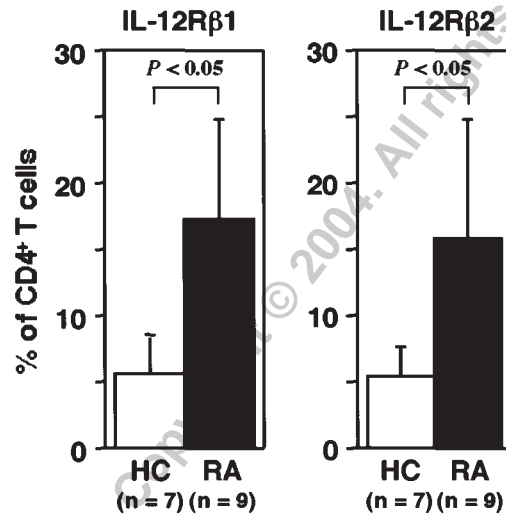
anti-human IL-12R $\beta$ 1 and  $\beta$ 2 mAb (2.4E6 and LM-5.2B6; Hoffmann La Roche, Nutley, NJ, USA) and mouse IgM anti-human IL-18R $\alpha$  mAb (117-10C; Hayashibara Biochemical Laboratories, Okayama, Japan), followed by fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat IgG2a mAb (PharMingen, San Diego, CA, USA) and phycoerythrin (PE)-conjugated rat anti-mouse IgM mAb (PharMingen), respectively. For control staining, FITC-conjugated rat IgG2a mAb (PharMingen) and PE-conjugated mouse IgM mAb (PharMingen) were used. PBMC were double-stained with PE-, FITC-, or peridium chlorophyll protein (PerCP)-conjugated anti-CD4 mAb (Leu-3a; Becton Dickinson, San Jose, CA, USA). Cells were incubated in the dark at 4°C with saturating concentrations of mAb in phosphate-buffered saline (PBS) with 1% FCS, and cells were washed well with 1% FCS/PBS between incubations. Analysis was performed using a FACScan cytometer (Becton Dickinson).

**Intracellular cytokine staining.** The production of IFN- $\gamma$  and IL-4 by ST CD4+ T cells was detected by intracellular cytokine staining, as described<sup>20</sup>. Briefly, CD4+ T cells from the ST were incubated with 50 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, MO, USA) and 1  $\mu$ g/ml calcium ionophore A23187 (Wako Pure Chemical, Osaka, Japan) in the presence of 10  $\mu$ g/ml brefeldin A (Sigma) for 4 h. Cells were stained with PerCP-anti-CD4 mAb (Becton Dickinson), mouse IgM anti-human IL-18R $\alpha$  mAb, and then PE-conjugated anti-mouse IgM mAb, or PE-control mAb (PharMingen), followed by fixation with 1% paraformaldehyde. Cells were then stained with FITC-anti-IFN- $\gamma$  mAb (4S.B3; PharMingen), FITC-anti-IL-4 mAb (MP4-25D2; PharMingen), and FITC-control mAb (PharMingen). After washing well with PBS/1% FCS with 0.5% saponin and then with PBS/1% FCS, cells were resuspended in PBS/1% FCS for flow cytometry.

**Isolation of mRNA and reverse transcriptase-polymerase chain reaction (RT-PCR).** Total cellular RNA was extracted from PB and ST CD4+ T cells using an RNA isolation kit (RNeasy Midi kit; Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The mRNA expression of IL-12R $\beta$ 1 and  $\beta$ 2 and IL-18R $\alpha$  and  $\beta$  was detected by RT-PCR, as described<sup>21</sup>. Briefly, complementary DNA (cDNA) was synthesized from total RNA with Molony murine leukemia virus reverse transcriptase (US Biochemical, Cleveland, OH, USA) and oligo-(dT)<sub>15</sub> primers (Promega, Madison, WI, USA). Samples of cDNA were PCR amplified for 35 cycles with *rTaq* DNA polymerase (Promega) and specific primers in a thermal cycler (iCycler<sup>TM</sup>, Bio-Rad Laboratories, Hercules, CA, USA). Each cycle consisted of 1 min of denaturation at 95°C and 2 min of annealing/extension at 65°C for  $\beta$ -actin at 60°C for IL-12R and IL-18R. PCR products were electrophoresed on 1.0% agarose gel and visualized by ethidium bromide staining. The sequences of oligonucleotide primers were as follows: for  $\beta$ -actin 5' primer GTGGGGCGCCCCAGGCACCA and 3' primer CTCCT-TAATGTCACGCACGATTTTC; for IL-12R $\beta$ 1 5' primer GCACCAC-GTCTCGGTGAAGA and 3' primer TAGTCTGTCTCTCGAGAG; for IL-12R $\beta$ 2 5' primer CCTAGAACC GGAAATTGGGC and 3' primer TTCCCTCTCATTTCCGTGGG; for IL-18R $\alpha$  5' primer GTTGAGT-TGAATGACACAGG and 3' primer TCCACTGCAACATGGTTAAG; and for IL-18R $\beta$  5' primer GATCAGACGCTTGGGGATAA and 3' primer TGTTTGATCATCCAAGGCAA.

## RESULTS

**Expression of IL-12R in RA PB CD4+ T cells.** PB CD4+ T cells in PBMC of RA patients and controls expressed negligible levels of IL-12R $\beta$ 1 and  $\beta$ 2 subunits on the cell surface ( $\beta$ 1 < 3.0%,  $\beta$ 2 < 1.0%). When PBMC were stimulated with immobilized anti-CD3 Ab, both IL-12R subunits were significantly induced on CD4+ T cells (Figure 1A). No significant difference was found in IL-12R expression between RA patients and controls (data not shown). Since monocyte products, such as IL-10 and transforming growth

**A****B**

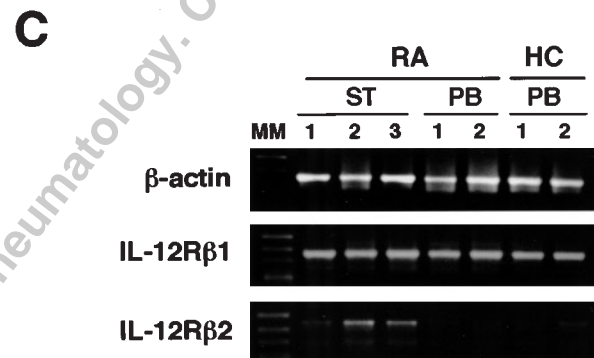
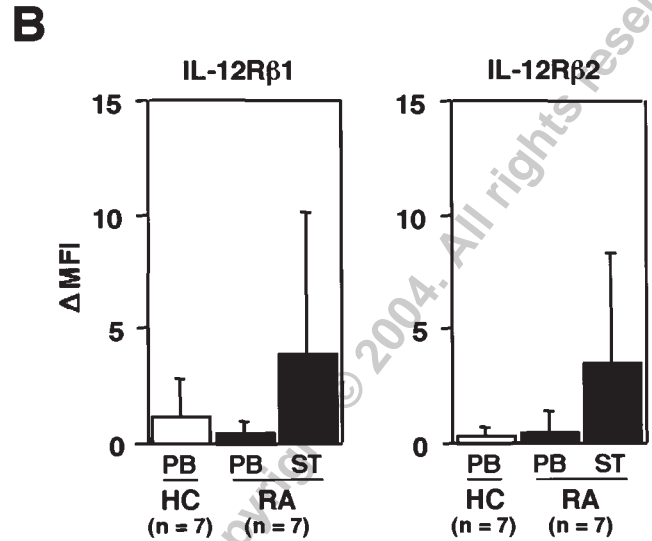
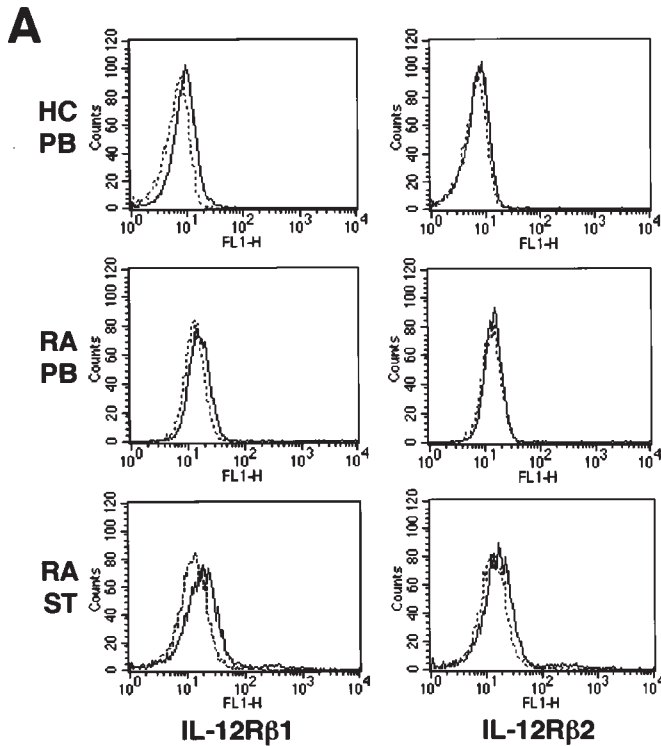
**Figure 1.** A. Expression of IL-12R β1 and β2 subunits on the cell surface of activated CD4+ T cells from patients with RA. PBMC  $1 \times 10^6$  cells/ml were incubated in 5% human serum/RPMI 1640 medium with immobilized anti-CD3 Ab (100 ng/ml), and were harvested 48 h later. Cells were stained with PerCP-conjugated anti-CD4 Ab and rat anti-IL-12Rβ1 Ab or anti-IL-12Rβ2 Ab, followed by FITC-conjugated mouse anti-rat IgG2a Ab, and cell surface expression of CD4 and IL-12Rβ1 and β2 were analyzed by flow cytometry. FITC-conjugated rat IgG2a Ab was used for control staining. Percentages of IL-12Rβ1+ and β2+ CD4+ T cells are indicated in the upper right corners. Results of one representative experiment are shown. B. Frequencies of IL-12Rβ1+ and β2+ cells in anti-CD3 Ab-activated CD4+ T cells from RA patients and healthy controls (HC). CD4+ T cells were purified from PBMC of RA patients and controls by positive selection with anti-CD4 Ab-coated magnetic beads. CD4+ T cells ( $2 \times 10^5$  cells/ml) were incubated with anti-CD3 Ab for 48 h, and percentages of IL-12Rβ1+ and β2+ cells were measured by flow cytometry. Values are expressed as mean  $\pm$  SD of the number of samples indicated. n = number of samples tested.

factor-β (TGF-β), have been shown to inhibit IL-12R expression<sup>8,22</sup>, purified CD4+ T cells from the PBMC of RA patients and controls were incubated for 48 h with anti-CD3 Ab and the percentage of IL-12R+ cells was measured by flow cytometric analysis. The induction of both IL-12Rβ1 and β2 expression on such activated CD4+ T cells was found at significantly higher levels in RA patients (mean  $\pm$  SD; β1  $17.2 \pm 7.7\%$ , β2  $15.8 \pm 9.1\%$ ) compared with controls (β1  $5.7 \pm 2.7\%$ , β2  $5.4 \pm 2.1\%$ ) (Figure 1B). These results indicate that PB CD4+ T cells in RA are highly capable and efficient IL-12R expressors.

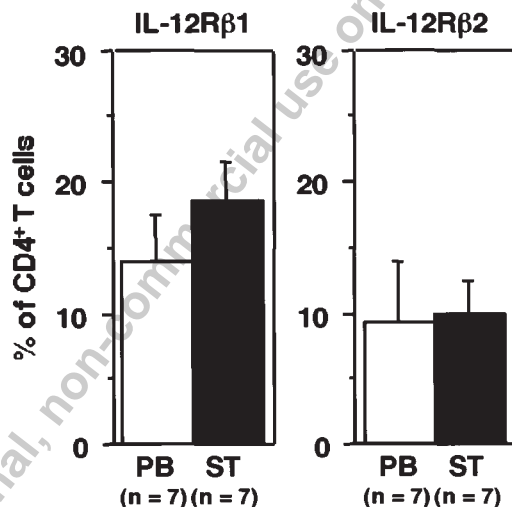
**Expression of IL-12R in RA ST CD4+ T cells.** The local expression of IL-12R in CD4+ T cells at the disease site of RA was examined by flow cytometry and RT-PCR. IL-12Rβ1 and β2 expression on freshly isolated ST CD4+ T

cells from RA patients was significantly detectable [ $\Delta$  mean fluorescence intensity (MFI): β1  $3.8 \pm 6.3$ , β2  $3.5 \pm 4.9$ ], whereas the expression on PB CD4+ T cells from the same patients (β1  $0.5 \pm 0.5$ , β2  $0.4 \pm 0.9$ ) or controls (β1  $1.2 \pm 1.6$ , β2  $0.4 \pm 0.4$ ) was insignificant (Figure 2A and 2B). The frequencies of IL-12Rβ1+ and β2+ CD4+ T cells in the ST were  $2.0 \pm 1.8\%$  and  $1.7 \pm 1.6\%$ , respectively. In addition, mRNA transcripts coding IL-12Rβ2 were more clearly detected in ST CD4+ T cells than in the PB cells of patients and controls, while IL-12Rβ1 transcripts were similarly detectable (Figure 2C). These results suggest that IL-12R expression could be induced locally in the ST, but the level of expression may be restricted to a greater extent due to the presence of inhibitors.

To determine the potential of ST CD4+ T cells to express



**Figure 2.** A. IL-12Rβ1 and β2 expression on freshly isolated peripheral blood (PB) and synovial tissue (ST) CD4+ T cells from RA patients. CD4+ T cells were purified from paired PB and ST samples of RA patients and PB samples of healthy controls (HC) by positive selection with anti-CD4 Ab-coated magnetic beads, and the intensities of IL-12Rβ1 and β2 expression were expressed as the difference between the mean fluorescence intensity (ΔMFI) of staining with anti-IL-12R Ab and isotype-matched control Ab. B. IL-12Rβ1 and β2 expression on fresh PB and ST CD4+ T cells from RA patients. The intensities of IL-12Rβ1 and β2 expression were expressed as ΔMFI. Values are expressed as mean ± SD of the number of samples indicated. n = number of samples tested. C. Transcriptional expression of IL-12Rβ1/β2 subunits in fresh PB and ST CD4+ T cells from RA patients. Total cellular RNA was extracted from purified PB and ST CD4+ T cells, and mRNA expression of IL-12Rβ1 and β2 was detected by RT-PCR, as described in Materials and Methods. MM = molecular weight markers.



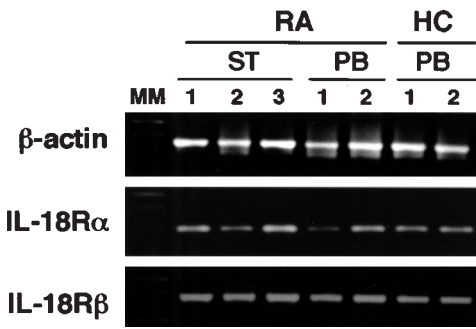
IL-12R, IL-12R induction after anti-CD3 Ab stimulation was compared between paired PB and ST CD4+ T cells of RA patients. There was no significant difference in the frequency of IL-12R+ cells between activated PB and ST CD4+ T cells (β1 14.0 ± 9.2% vs 18.5 ± 7.9%, β2 9.3 ± 12.3% vs 9.9 ± 6.7%) (Figure 3), with a positive correlation between PB and ST IL-12Rβ2+ cells ( $r = 0.801$ ,  $p = 0.027$ ). Thus, the capacity of CD4+ T cells for IL-12R expression seems to be maintained after entry into the ST.

*Expression of IL-18R in RA PB and ST CD4+ T cells.* The

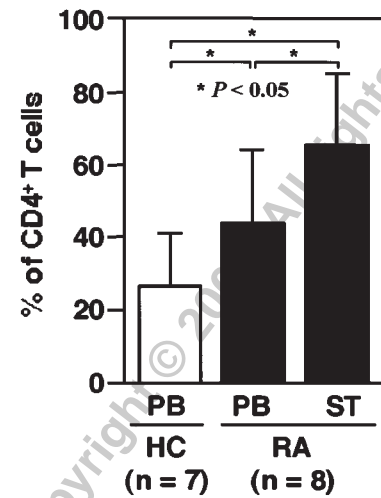
**Figure 3.** Frequencies of IL-12Rβ1+ and β2+ cells in anti-CD3 Ab-activated PB and ST CD4+ T cells from RA patients. Purified CD4+ T cells ( $2 \times 10^5$  cells/ml) from paired PB and ST samples of RA patients were incubated with anti-CD3 Ab for 48 h, and the percentages of IL-12Rβ1+ and β2+ cells were measured by flow cytometry. Values are expressed as mean ± SD of the number of samples indicated.



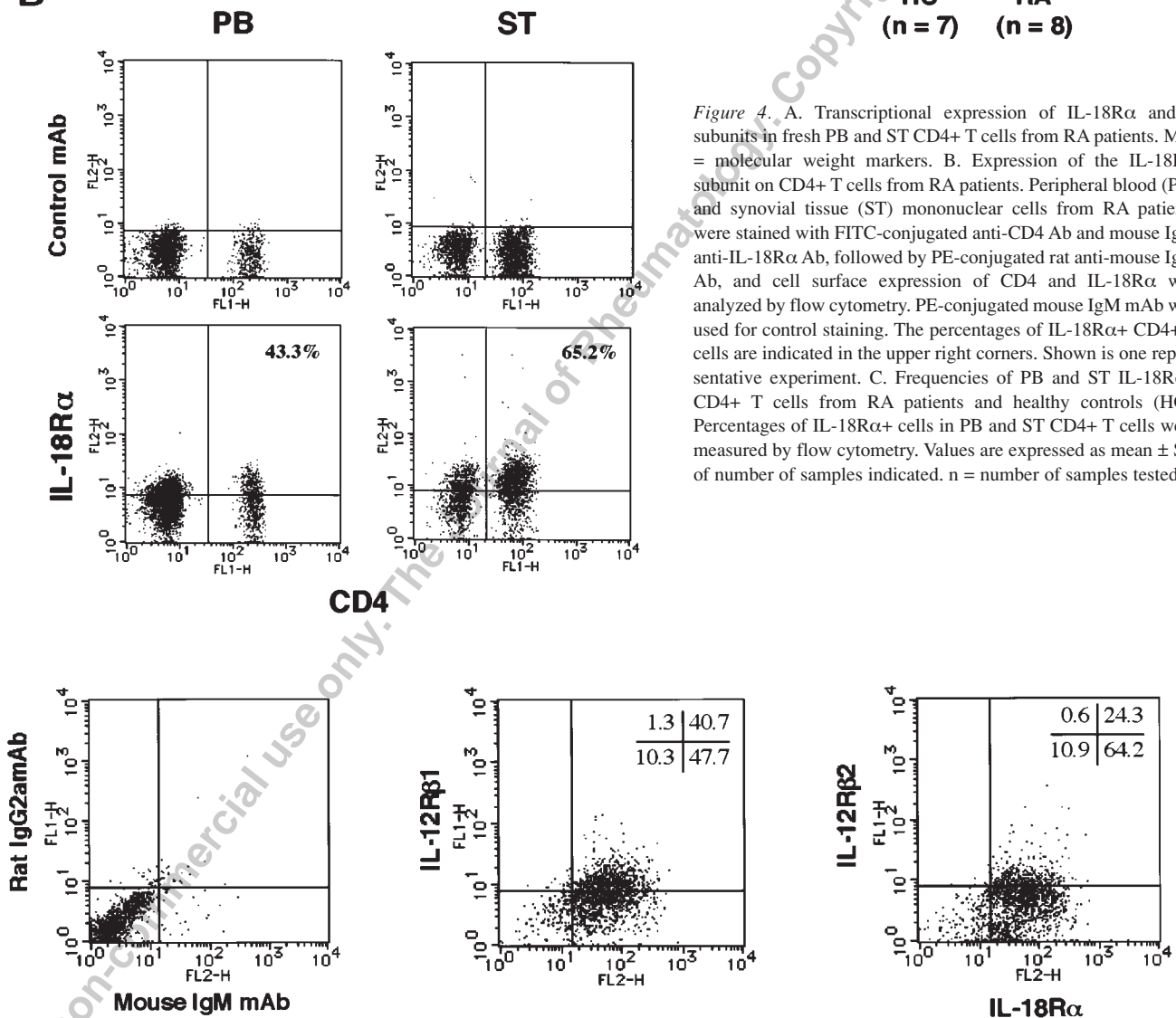
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**C**

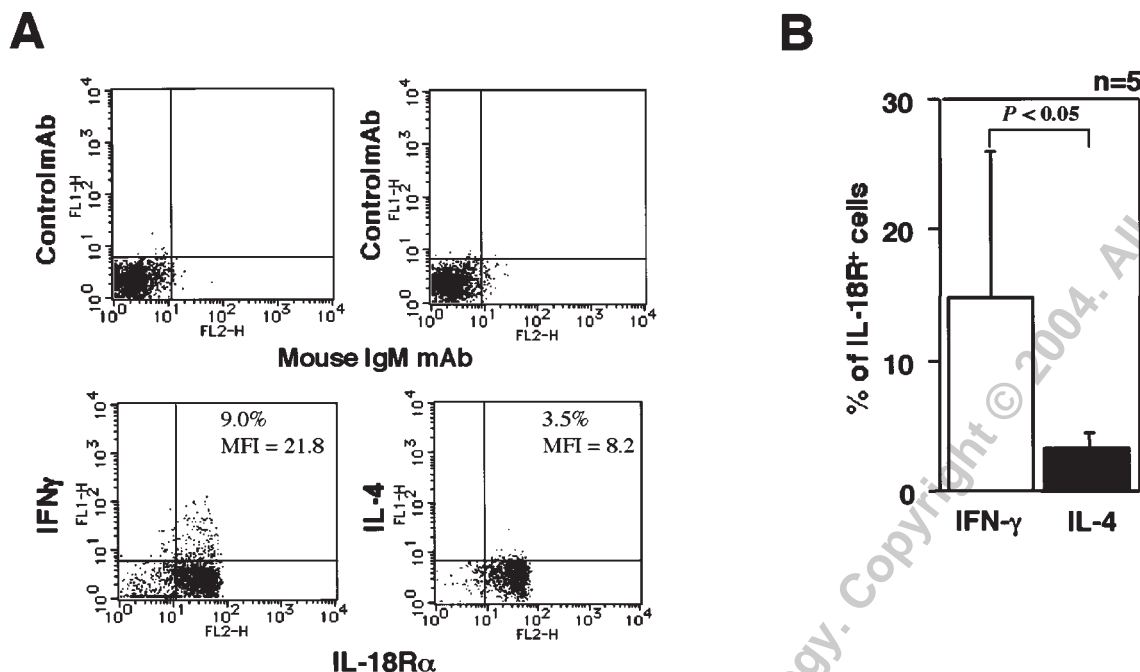


**B**



*Figure 4. A. Transcriptional expression of IL-18R $\alpha$  and  $\beta$  subunits in fresh PB and ST CD4+ T cells from RA patients. MM = molecular weight markers. B. Expression of the IL-18R $\alpha$  subunit on CD4+ T cells from RA patients. Peripheral blood (PB) and synovial tissue (ST) mononuclear cells from RA patients were stained with FITC-conjugated anti-CD4 Ab and mouse IgM anti-IL-18R $\alpha$  Ab, followed by PE-conjugated rat anti-mouse IgM Ab, and cell surface expression of CD4 and IL-18R $\alpha$  was analyzed by flow cytometry. PE-conjugated mouse IgM mAb was used for control staining. The percentages of IL-18R $\alpha$ + CD4+ T cells are indicated in the upper right corners. Shown is one representative experiment. C. Frequencies of PB and ST IL-18R $\alpha$ + CD4+ T cells from RA patients and healthy controls (HC). Percentages of IL-18R $\alpha$ + cells in PB and ST CD4+ T cells were measured by flow cytometry. Values are expressed as mean  $\pm$  SD of number of samples indicated. n = number of samples tested.*

*Figure 5. Induction of IL-12R $\beta$ 1 and  $\beta$ 2 expression on anti-CD3 antibody (Ab)-activated synovial tissue (ST) CD4+ T cells from RA patients. Purified ST CD4+ T cells ( $2 \times 10^5$  cells/ml) from RA patients were incubated with anti-CD3 Ab for 48 h, and cell surface expression of IL-12R $\beta$ 1 and  $\beta$ 2 and IL-18R $\alpha$  was determined by flow cytometry. Percentages of cells in each quadrant are indicated in the upper right corners. One representative result from 3 experiments with different patients in which similar results were obtained is shown.*



**Figure 6.** IFN- $\gamma$  and IL-4 production by synovial tissue (ST) IL-18R $\alpha$ + CD4+ T cells from RA patients. A. Purified ST CD4+ T cells from RA patients ( $2 \times 10^5$  cells/ml) were stimulated with PMA and calcium ionophore A23187 in the presence of brefeldin A for 4 h. Cells were stained with anti-IL-18R $\alpha$  Ab, and then stained with FITC-conjugated anti-IFN- $\gamma$  Ab or anti-IL-4 Ab. Expression of IL-18R $\alpha$ , IFN- $\gamma$ , and IL-4 was analyzed by flow cytometry. Percentages and mean fluorescence intensity (MFI) of IFN- $\gamma$ - and IL-4-producing cells are indicated in the upper right corners. One representative result from 5 experiments with different patients in which similar results were obtained is shown. B. Percentages of IFN- $\gamma$ - and IL-4-producing cells in IL-18R $\alpha$ + CD4+ T cells from 5 RA patients are shown. Values are expressed as mean  $\pm$  SD of the number of samples indicated.

expression of IL-18R in PB and ST CD4+ T cells from RA patients and PB CD4+ T cells from controls was examined by RT-PCR and flow cytometry. Both IL-18R $\alpha$  and  $\beta$  mRNA transcripts were detected in RA PB and ST CD4+ T cells, as well as control PB CD4+ T cells (Figure 4A). By flow cytometric analysis, the IL-18R $\alpha$  subunit was found to be significantly expressed on the cell surface of both CD4+ and CD4- cells in the lymphocyte gate of PBMC and ST cells (Figure 4B). The frequency of IL-18R $\alpha$ + CD4+ T cells was significantly higher in RA PB ( $40.4 \pm 18.5\%$ ) than in control PB ( $26.9 \pm 14.3\%$ ), and was further increased in the ST ( $62.5 \pm 17.4\%$ ) (Figure 4C). These results indicate that IL-18R $\alpha$ + CD4+ T cells are accumulated in the ST lesion due to their preferential recruitment and/or local expansion.

**Expression of IL-12 $\beta$ 1 and  $\beta$ 2 on RA ST IL-18R $\alpha$ + CD4+ T cells.** To examine whether IL-12R is induced in activated IL-18R $\alpha$ + CD4+ T cells, the expression of these cytokine receptors on ST CD4+ T cells from RA patients after anti-CD3 Ab stimulation was determined by flow cytometry. As shown in Figure 5, both IL-12R $\beta$ 1 and  $\beta$ 2 subunits were found to be expressed largely on IL-18R $\alpha$ + CD4+ T cells.

**IFN- $\gamma$  production by IL-18R $\alpha$ -expressing RA ST CD4+ T cells.** Previous studies have indicated that the IL-18R $\alpha$  subunit is selectively expressed on Th1 cells<sup>15,16</sup>. ST CD4+ T cells were stimulated with PMA and A23187, and the

**Table 1.** Augmentation of IL-12R $\beta$ 1 and  $\beta$ 2 expression by IL-18 in anti-CD3 antibody (Ab)-activated CD4+ T cells from synovial tissue (ST) of patients with RA. CD4+ T cells were purified from ST samples of patients with RA by positive selection with anti-CD4 Ab-coated magnetic beads. CD4+ T cells ( $2 \times 10^5$  cells/ml) were incubated in 5% human serum/RPMI 1640 medium with immobilized anti-CD3 Ab (100 ng/ml) with or without IL-18 (1 ng/ml), and were harvested 48 h later. Percentages of IL-12R $\beta$ 1+ and  $\beta$ 2+ CD4+ T cells were determined by flow cytometry.

	Experiment		
	1	2	3
IL-12R $\beta$ 1, %			
Anti-CD3	13.0	17.7	6.3
Anti-CD3 + IL-18	20.2	23.4	16.1
IL-12R $\beta$ 2, %			
Anti-CD3	8.6	3.1	4.5
Anti-CD3 + IL-18	12.0	7.7	9.6

expression of IL-18R $\alpha$ , IFN- $\gamma$ , and IL-4 was determined by intracellular cytokine staining and flow cytometry. As shown in Figure 6A, stimulation of PMA and A23187 resulted in increased IL-18R $\alpha$  expression, and these IL-18R+ CD4+ T cells produced high levels of IFN- $\gamma$  but low levels of IL-4. The frequencies of IFN- $\gamma$ - and IL-4-producing cells in ST IL-18R $\alpha$ + CD4+ T cells were  $14.8 \pm 11.2\%$  and  $3.2 \pm 1.0\%$ , respectively (Figure 6B). These

results indicate that ST CD4+ T cells can mostly express IL-12R when activated, and they are potential IFN- $\gamma$  producers. *IL-12R induction by IL-18 in RA ST CD4+ T cells.* IL-18 has been shown to upregulate IL-12R expression on activated CD4+ T cells<sup>23</sup>. ST CD4+ T cells of RA patients were stimulated with anti-CD3 Ab with or without IL-18, and IL-12R expression was determined by flow cytometry. The frequency of both IL-12R $\beta$ 1+ and  $\beta$ 2+ in anti-CD3 Ab-activated CD4+ T cells was augmented by the addition of IL-18 (Table 1). Given the high concentration of IL-18 in the joints<sup>4,5</sup>, this cytokine may be an important inducer of local IL-12R expression in RA.

## DISCUSSION

IL-12 can directly induce the selective synthesis of IFN- $\gamma$  in T cell infiltrates in RA ST, but the local expression of this dominant factor is limited<sup>3</sup>. In contrast, IL-18 is abundantly produced by tissue macrophages, but the direct effect of this proinflammatory cytokine on IFN- $\gamma$  production by ST T cells is less potent<sup>4,5</sup>. The synergistic effect of IL-12 and IL-18 is thus considered to be crucial in perpetuating the IFN- $\gamma$ -dominant T cell response of RA.

We demonstrated that, although both IL-12R subunits are not readily detectable on fresh cells, PB CD4+ T cells from RA patients are induced to express higher concentrations of IL-12R $\beta$ 1 and  $\beta$ 2 after stimulation with anti-CD3 Ab, compared with the cells of controls. IL-12R $\beta$ 1 and  $\beta$ 2 expression by ST CD4+ T cell infiltrates is detectable but limited. In contrast, the inducible IL-18R $\alpha$  subunit is more significantly expressed on PB CD4+ T cells in RA patients than in controls, and IL-18R $\alpha$ + CD4+ T cells are highly enriched in the ST. When ST CD4+ T cells are activated with anti-CD3 Ab, both IL-12R $\beta$ 1 and  $\beta$ 2 are induced mainly on IL-18R $\alpha$ + cells. After PMA and A27187 stimulation, ST CD4+ T cells mostly express IL-18R $\alpha$  and predominantly produce IFN- $\gamma$ . IL-18 augments IL-12R expression on anti-CD3 Ab-activated ST CD4+ T cells. Taken together, these findings indicate that the functional IL-12R is induced in IL-18R+ CD4+ T cell infiltrates by stimuli such as CD3 activation and IL-18, and the expression of both cytokine receptors may be essential for local IFN- $\gamma$  synthesis in RA.

Cytokine expression by T cell infiltrates in RA is characterized by predominance of the Th1 cytokine IFN- $\gamma$  over the Th2 cytokine IL-4<sup>1</sup>. We have demonstrated with intracellular cytokine staining that the majority of ST CD4+ T cells are able to produce IFN- $\gamma$  after stimulation with PMA and A23187<sup>20</sup>. However, immunostaining of RA tissues revealed the low-level expression of IFN- $\gamma$  in the ST, with up to 0.5% of the total T cells expressing IFN- $\gamma$ <sup>24,25</sup>. This discrepancy indicates that CD4+ Th1 cells are selectively accumulated, with very few Th2 cells, but only a small proportion of Th1 cells are fully activated in the ST.

The IL-12R $\beta$ 2 subunit, an essential component of the signaling pathway leading to STAT4 activation, is selec-

tively expressed in Th1 clones and established Th1 cells<sup>26,27</sup>. IL-12R $\beta$ 2 expression by ST CD4+ T cells was detected at low levels, but was highly increased after CD3 activation. This observation is likely consistent with the discrepancy between *in vitro* and *in vivo* IFN- $\gamma$  expression in ST T cells as described above. The limited activation of Th1 cells *in vivo* may be attributable to an abundance of immunoregulatory cytokines such as IL-10 and TGF- $\beta$  in RA joints<sup>24</sup>, in addition to the restricted antigen-dependent T cell activation *in vivo*<sup>28</sup>. In this regard, these cytokines have been shown to potently suppress IL-12R expression<sup>8,22</sup> as well as IL-12 production<sup>6</sup>. The level of IL-12R detected at the disease site, although limited, is believed to have pathogenic significance in RA, based on the observation that a high level of IFN- $\gamma$  production by cultured ST T cells could be induced by IL-12 alone<sup>3</sup>. These findings suggest that CD4+ T cells expressing both IL-12R subunits may represent the functionally activated Th1 cells.

IL-12 is well known as a critical factor in polarizing the T cell response toward a Th1 type<sup>6,7</sup>, but IL-18 stimulates both Th1 and Th2 responses<sup>14</sup>. In the presence of IL-12, IL-18 has been shown to enhance the Th1-type immune response<sup>29,30</sup>. The reciprocal upregulation of their receptors on T cells has been demonstrated as one potential basis for this synergism<sup>31-35</sup>. The functional IL-12R, particularly the  $\beta$ 2 subunit, appears to be induced in IL-18R $\alpha$ + CD4+ T cells after their entry into the ST, in the inflammatory microenvironment of RA. Our finding of efficient IL-12R induction by costimulation with anti-CD3 Ab and IL-18 in ST CD4+ T cells indicates that IL-18 may be an important inducer for local IL-12R expression. However, because of the paucity of IL-12 in the joints and the rarity of IL-12R+ CD4+ T cells in ST, IL-18R upregulation by IL-12 may be less significant in RA.

For formation of functional high-affinity IL-18R, coexpression of the  $\alpha$  and  $\beta$  subunits is required<sup>14</sup>. IL-18R $\beta$  is ubiquitously expressed on T cells<sup>33</sup>, but the signal-transducing IL-18R $\alpha$  is selectively expressed on differentiated Th1 cells<sup>15,16</sup>, indicating the relevance of IL-18R $\alpha$  to Th1 cell effector function. Instead of IL-18R $\alpha$ , Th2 cells have recently been shown to selectively express the human homologue of the ST2L molecule known as a stable marker of murine Th2 cells<sup>16</sup>. PB CD4+ T cells from RA patients contained mRNA transcripts for both subunits, and their protein expression of the  $\alpha$  subunit was increased compared with control cells. This finding may be consistent with the systemic shift toward Th1 response in RA, as indicated<sup>36,37</sup>.

IL-18R $\alpha$ + CD4+ T cells are enriched in the ST lesion. This accumulation may be due in part to the local activation of T cells, because ST CD4+ T cells activated *in vitro* showed both increased IL-18R $\alpha$  expression and predominant IFN- $\gamma$  production. More importantly, IL-18R $\alpha$ + Th1 cells are believed to be preferentially recruited from PB into

the ST. Recent evidence indicates that the selective infiltration of Th1 cells into the site of inflammation is mediated by specific interactions between Th1 cell-specific chemokine receptors and their corresponding chemokines. In RA, the majority of T cell infiltrates express the chemokine receptors CCR5 and CXCR3<sup>38,39</sup>, and their agonistic ligands are present at high concentrations in the joints, including monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 (MIP-1), regulated upon activation normal T cell expressed and secreted (RANTES), monokine induced by IFN- $\gamma$  (MIG), IFN- $\gamma$ -inducible T cell  $\alpha$  chemoattractant (ITAC), and IFN-inducible protein-10 (IP-10)<sup>39</sup>. Both the local activation and selective recruitment of IL-18R $\alpha$ + CD4+ Th1 cells could be responsible for their accumulation in the ST lesion.

Coexpression of IL-12R and IL-18R is required for maximal transcriptional activation of the IFN- $\gamma$  gene. Upon binding of these receptors, IL-12 and IL-18 differentially activate the IFN- $\gamma$  promoter. IL-12 directly activates the transcription factor STAT4<sup>26</sup>, whereas IL-18 induces the activity of NF- $\kappa$ B and AP-1<sup>34,40,41</sup>. The combined effect of these transcription factors can effectively activate the IFN- $\gamma$  promoter<sup>42</sup>, which is known as another basis for the synergism of IL-12 and IL-18.

In summary, IL-18R $\alpha$ + CD4+ T cells are accumulated into the ST of patients with RA, where IL-12R induction may be augmented by an abundance of IL-18. Coexpression of IL-12R and IL-18R may be a prerequisite for the IFN- $\gamma$ -dominant cytokine response in RA, because the stimulatory concentration of IL-12 is low and IFN- $\gamma$  induction by IL-18 is largely IL-12-dependent in the ST. The synergistic effect of IL-12 and IL-18 could be achieved by simultaneous activation of several transcription factors induced by their receptor-mediated signaling.

## REFERENCES

- Miossec P, van den Berg W. Th1/Th2 cytokine balance in arthritis. *Arthritis Rheum* 1997;40:2105-15.
- Choy EHS, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med* 2001;344:907-16.
- Morita Y, Yamamura M, Nishida K, et al. Expression of interleukin-12 in synovial tissue from patients with rheumatoid arthritis. *Arthritis Rheum* 1998;41:306-13.
- Gracie JA, Forsey RJ, Chan WL, et al. A proinflammatory role for IL-18 in rheumatoid arthritis. *J Clin Invest* 1999;104:1393-401.
- Yamamura M, Kawashima M, Tani M, et al. Interferon- $\gamma$ -inducing activity of interleukin-18 in the joint with rheumatoid arthritis. *Arthritis Rheum* 2001;44:275-85.
- Gately MK, Renzetti LM, Magram J, et al. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol* 1998;16:495-521.
- Sinigaglia F, D'Ambrosio D, Panina-Bordignon P, Rogge L. Regulation of the IL-12/IL-12R axis: a critical step in T-helper cell differentiation and effector function. *Immunol Rev* 1999;170:65-72.
- Zhang M, Gong J, Presky DH, Xue W, Barnes PF. Expression of the IL-12 receptor  $\beta$ 1 and  $\beta$ 2 subunits in human tuberculosis. *J Immunol* 1999;162:2441-7.
- Rogge L, Papi A, Presky DH, et al. Antibodies to the IL-12 receptor  $\beta$ 2 chain mark human Th1 but not Th2 cells in vitro and in vivo. *J Immunol* 1999;162:3926-32.
- Kim J, Uyemura K, Van Dyke MK, et al. A role for IL-12 receptor expression and signal transduction in host defense in leprosy. *J Immunol* 2001;167:779-86.
- Yamasaki H, Ando M, Brazer W, et al. Polarized type 1 cytokine profile in bronchoalveolar lavage T cells of patients with hypersensitivity pneumonitis. *J Immunol* 1999;163:3516-23.
- Parrello T, Monteleone G, Cucchiara S, et al. Up-regulation of the IL-12 receptor  $\beta$ 2 chain in Crohn's disease. *J Immunol* 2000;165:7234-9.
- Ozenci V, Pashenkov M, Kouwenhoven, et al. IL-12/IL-12R system in multiple sclerosis. *J Neuroimmunol* 2001;114:242-52.
- Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* 2001;19:423-74.
- Xu D, Chan WL, Leung BP, et al. Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. *J Exp Med* 1998;188:1485-92.
- Chan WL, Pejnovic N, Lee CA, Al-Ali NA. Human IL-18 receptor and ST2L are stable and selective markers for the respective type 1 and type 2 circulating lymphocytes. *J Immunol* 2001;167:1238-44.
- Greene CM, Meachery G, Taggart CC, et al. Role of IL-18 in CD4+ T lymphocyte activation in sarcoidosis. *J Immunol* 2000;165:4718-24.
- Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
- Okamoto H, Yamamura M, Morita Y, et al. The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis. *Arthritis Rheum* 1997;40:1096-105.
- Morita Y, Yamamura M, Kawashima M, et al. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum* 1998;41:1669-76.
- Yamamura M, Uyemura K, Deans RJ, et al. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* 1991;254:277-9.
- Wu C, Warrier RR, Wang X, et al. Regulation of interleukin-12 receptor  $\beta$ 1 chain expression and interleukin-12 binding by human peripheral blood mononuclear cells. *Eur J Immunol* 1997;27:147-54.
- Chang JT, Segal BM, Nakanishi K, Okamura H, Shevach EM. The costimulatory effect of IL-18 on the induction of antigen-specific IFN- $\gamma$  production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor  $\beta$ 2 subunit. *Eur J Immunol* 2000;30:1113-9.
- Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* 1996;14:397-440.
- Cohen SB, Katsikis PD, Chu CQ, et al. High level of interleukin-10 production by the activated T cell population within the rheumatoid synovial membrane. *Arthritis Rheum* 1995;38:946-52.
- Rogge L, Barberis-Maino L, Biffi M, et al. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J Exp Med* 1997;185:825-31.
- Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 1997;185:817-24.
- Panayi GS, Lanchbury JS, Kingsley GH. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis Rheum* 1992;35:729-35.
- Micallef MJ, Ohtsuki T, Kohno K, et al. Interferon- $\gamma$ -inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon- $\gamma$



- production. *Eur J Immunol* 1996;26:1647-51.
30. Kohno K, Kataoka J, Ohtsuki T, et al. IFN- $\gamma$ -inducing factor is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12. *J Immunol* 1997;158:1541-50.
  31. Yoshimoto T, Takeda K, Tanaka T, et al. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN- $\gamma$  production. *J Immunol* 1998;161:3400-7.
  32. Kunikata T, Torigoe K, Ushio S, et al. Constitutive and induced IL-18 receptor expression by various peripheral blood cell subsets as determined by anti-hIL-18R monoclonal antibody. *Cell Immunol* 1998;189:135-43.
  33. Tominaga K, Yoshimoto T, Torigoe K, et al. IL-12 synergizes with IL-18 or IL-1 $\beta$  for IFN- $\gamma$  production from human T cells. *Int Immunol* 2000;12:151-60.
  34. Sareneva T, Julkunen I, Matikainen S. IFN- $\gamma$  and IL-12 induce IL-18 receptor gene expression in human NK and T cells. *J Immunol* 2000;165:1933-8.
  35. Kim SH, Reznikov LL, Stuyt RJ, et al. Functional reconstitution and regulation of IL-18 activity by the IL-18R  $\beta$  chain. *J Immunol* 2001;166:148-54.
  36. Schulze-Koops H, Davis LS, Haverty TP, Wacholtz MC, Lipsky PE. Reduction of Th1 cell activity in the peripheral circulation of patients with rheumatoid arthritis after treatment with a non-depleting humanized monoclonal antibody to CD4. *J Rheumatol* 1998;25:2065-76.
  37. Wong WM, Vakis SA, Ayre KR, et al. Rheumatoid arthritis T cells produce Th1 cytokines in response to stimulation with a novel trispecific antibody directed against CD2, CD3, and CD28. *Scand J Rheumatol* 2000;29:282-7.
  38. Loetscher P, Uguccioni M, Bordoli L, et al. CCR5 is characteristic of Th1 lymphocytes. *Nature* 1998;391:344-5.
  39. Patel DD, Zachariah JP, Whichard LP. CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. *Clin Immunol* 2001;98:39-45.
  40. Matsumoto S, Tsuji-Takayama K, Aizawa Y, et al. Interleukin-18 activates NF- $\kappa$ B in murine T helper type 1 cells. *Biochem Biophys Res Commun* 1997;234:454-7.
  41. Sareneva T, Julkunen I, Matikainen S. IFN- $\gamma$  and IL-12 induce IL-18 receptor gene expression in human NK and T cells. *J Immunol* 2000;165:1933-8.
  42. Murphy KM, Ouyang W, Farrar JD, et al. Signaling and transcription in T helper development. *Annu Rev Immunol* 2000;18:451-94.