

Primary research

Cytokine, activation marker, and chemokine receptor expression by individual CD4⁺ memory T cells in rheumatoid arthritis synovium

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Statement of findings

IL-10, IL-13, IFN- γ , tumor necrosis factor (TNF)- α , LT- α , CD154, and TNF-related activation-induced cytokine (TRANCE) were expressed by 2–20% of rheumatoid arthritis (RA) synovial tissue CD4⁺ memory T cells, whereas CD4⁺ cells that produced IL-2, IL-4, or IL-6 were not detected. Expression of none of these molecules by individual CD4⁺ cells correlated with the exception of TRANCE and IL-10, and TRANCE and TNF- α . A correlation between expression of IL-10 and CCR7, LT- α and CCR6, IFN- γ and CCR5, and TRANCE and CXCR4 was also detected.

Keywords: chemokine receptor, cytokine, rheumatoid arthritis, T lymphocyte

Synopsis

Introduction: In RA large numbers of CD4⁺ memory T cells infiltrate the inflamed synovium [1–3]. The accumulated CD4⁺ memory T cells in the RA synovium appear to be activated, because they express cytokines and activation markers [4–8]. Expressed cytokines and activation markers should play important roles in the pathogenesis of RA. However, the frequency of cytokine expression by RA synovial CD4⁺ T cells has not been analyzed accurately. Recently, the roles of chemokine and chemokine receptor interactions in T-cell migration have been intensively examined. Interactions of chemokine and chemokine receptors might therefore be important in the accumulation of the CD4⁺ T cells in the RA synovium. Accordingly, correlation of cytokine and chemokine receptor expression might be important in delineating the function and potential means of accumulation of individual CD4⁺ memory T cells in the RA synovium.

In the present study we analyzed cytokine (IL-2, IL-4, IL-6, IL-10, IL-13, IFN- γ , TNF- α , and LT- α), activation marker (CD154 [CD40 ligand] and TRANCE – also called receptor activator of nuclear factor κ B ligand [RANKL] or osteoclast

differentiation factor [ODF]), and chemokine receptor expression by individual CD4⁺ memory T cells isolated from rheumatoid synovium and blood. To achieve this we employed a single-cell reverse transcription (RT) polymerase chain reaction (PCR) technique. This technique made it possible to correlate mRNAs expressed by individual CD4⁺ memory T cells in the synovium and blood.

Materials and method: Synovial tissues from three RA patients and peripheral blood mononuclear cells from two RA patients and a normal donor were analyzed.

Cytokine (IL-2, IL-4, IL-6, IL-10, IL-13, IFN- γ , TNF- α , and LT- α) and activation marker (CD154 and TRANCE) expression by individual CD4⁺CD45RO⁺ T cells from RA synovium or blood were analyzed using a single-cell RT-PCR. In brief, single CD4⁺CD45RO⁺ T cells was sorted into each well of a 96-well PCR plate using a flow cytometer. cDNA from individual cells was prepared, and then the cDNA was nonspecifically amplified. The product was then amplified by PCR using gene-specific primers to analyze cytokine and activation marker expression.

Table 1

Frequencies of cytokine and activation marker expression by CD4⁺DC45RO⁺ T cells in synovial tissue of RA patients

	IL-2	IL-4	IL-6	IL-10	IL-13	IFN- γ	TNF- α	LT- α	CD154	TRANCE
RA1 (n = 50)	0 (0)	0 (0)	0 (0)	7 (14)	0 (0)	11 (22)	0 (0)	6 (12)	7 (14)	8 (16)
RA2 (n = 39)	0 (0)	0 (0)	0 (0)	5 (13)	1 (3)	3 (8)	0 (0)	1 (3)	10 (26)	1 (3)
RA3 (n = 63)	0 (0)	0 (0)	0 (0)	4 (6)	4 (6)	4 (6)	3 (5)	4 (6)	14 (22)	3 (5)
Total (n = 152)	0 (0)	0 (0)	0 (0)	16 (11)	5 (3)	18 (12)	3 (2)	11 (7)	31 (20)	12 (8)

Values are expressed as number (percentage) of cytokine-positive cells in TCR C β -positive cells.

Table 2

Correlation of cytokine expression in 152 individual RA synovial tissue CD4⁺ memory T cells

	IL-13	IFN- γ	TNF- α	LT- α	CD154	TRANCE	Total
IL-10 (+)	0 (0)	2 (13)	0 (0)	2 (13)	4 (25)	4 (25)*	16
IL-10 (-)	5 (4)	16 (12)	3 (2)	9 (7)	27 (20)	8 (6)	136
IL-13 (+)		1 (20)	1 (20)	0 (0)	0 (0)	1 (20)	5
IL-13 (-)		17 (12)	2 (1)	11 (7)	31 (21)	11 (7)	147
IFN- γ (+)			0 (0)	0 (0)	4 (22)	3 (17)	18
IFN- γ (-)			3 (2)	11 (8)	27 (20)	9 (7)	134
TNF- α (+)				0 (0)	0 (0)	2 (67)*	3
TNF- α (-)				11 (7)	31 (21)	10 (7)	149
LT- α (+)					4 (36)	1 (9)	11
LT- α (-)					27 (19)	11 (8)	141
CD154 (+)						1 (3)	31
CD154 (-)						11 (9)	121

Values are expressed as number (percentage) of cytokine-positive cells. *P < 0.05.

Results: Cytokine and activation marker expression by individual CD4⁺CD45RO⁺ T cells from RA synovial tissues was analyzed using a single-cell RT-PCR method. Expression of mRNAs was analyzed in 152 individual synovial tissue CD4⁺CD45RO⁺ T cells sorted from three RA patients in which T-cell receptor (TCR) C β mRNA was detected. Frequencies of CD4⁺ memory T cells expressing cytokine and activation marker mRNA in RA synovium are shown in Table 1. IL-2, IL-4, and IL-6 were not expressed by the synovial tissue CD4⁺CD45RO⁺ T cells, whereas 2–20% of cells expressed the other cytokine mRNAs. Few correlations between cytokine and activation marker mRNAs were observed. Notably, no cells contained both IFN- γ and LT- α mRNAs, cytokines that are thought to define the T-helper (Th)1 phenotype [9]. However, the frequency of TRANCE-positive cells in IL-10-positive cells was significantly higher than that in IL-10-negative cells (Table 2). Moreover, the frequency of TRANCE-positive cells in TNF- α -positive cells was also significantly higher than that in TNF- α -negative cells. Varying percentages of CD4⁺ memory T cells expressed CC and CXC chemokine receptors. The frequency of CCR5-positive cells in IFN- γ -positive cells was significantly higher than

that in IFN- γ -negative cells, whereas the frequency of CCR6-positive cells in LT- α -positive cells was significantly higher than that in LT- α -negative cells, and the frequency of CCR7-positive cells in IL-10-positive cells was significantly higher than that in IL-10-negative cells. Furthermore, the frequency of CXCR4-positive cells in TRANCE-positive cells was significantly higher than that in TRANCE-negative cells.

Expression of cytokine and activation marker mRNAs was also analyzed in 48 individual peripheral blood CD4⁺CD45RO⁺ T cells from two RA patients. IL-2, IL-4, IL-6, and LT- α were not expressed by the peripheral CD4⁺CD45RO⁺ T cells, whereas 4–17% of cells expressed the other markers. The most striking difference between synovial tissue and peripheral blood CD4⁺ memory T cells was the presence of LT- α expression in the former, but not in the latter. IFN- γ and TNF- α were not expressed by normal peripheral blood CD4⁺ memory T cells, although they were expressed by RA peripheral blood CD4⁺ memory T cells.

Discussion: The present study employed a single-cell PCR technology to analyze cytokine expression by unstimulated RA synovial tissue CD4⁺ memory T cells immediately after isolation, without *in vitro* manipulation. The results confirm the

Th1 nature of rheumatoid inflammation. It is noteworthy that no individual synovial CD4⁺ memory T cells expressed both IFN- γ and LT- α mRNAs, even though these are the prototypic Th1 cytokines [9]. These results imply that, in the synovium, regulation of IFN- γ and LT- α must vary in individual cells, even though both Th1 cytokines can be produced.

The present data showed that CCR5 expression correlated with IFN- γ but not with LT- α expression by synovial CD4⁺ memory T cells. It has been reported that CCR5 expression is upregulated in RA synovial fluid and synovial tissue T cells [10–12] and that CCR5 Δ 32 deletion may have an influence on clinical manifestations of RA [13], suggesting that CCR5 might play an important role in RA. Recently, it has been claimed that CCR5 was preferentially expressed by Th1 cell lines [14,15]. However, in the present study CCR5 was not expressed by all IFN- γ -expressing cells. Moreover, CCR5 expression did not correlate with expression of LT- α by RA synovial CD4⁺ memory T cells. Therefore, it is unclear whether CCR5 is a marker of Th1 cells in RA synovium.

IL-10 expression correlated with CCR7 expression by RA synovial CD4⁺ memory T cells. Recently, it was reported [16] that in the blood CCR7⁺ CD4⁺ memory T cells express lymph-node homing receptors and lack immediate effector function, but efficiently stimulate dendritic cells. These cells may play a unique role in the synovium as opposed to in the blood. By producing IL-10, they might have an immunoregulatory function. In addition, IL-10 expression also correlated with expression of TRANCE. Although it is possible that IL-10 produced by these cells inhibited T-cell activation in the synovium, TRANCE expressed by these same cells might function to activate dendritic cells and indirectly stimulate T cells, mediating inflammation in the synovium. These results imply that individual T cells in the synovium might have different, and sometimes opposite functional activities.

LT- α expression correlated with CCR6 expression by synovial CD4⁺ memory T cells. It has been reported that CCR6 is expressed by resting peripheral memory T cells [17], whereas LT- α expression is associated with the presence of lymphocytic aggregates in synovial tissue [7]. The correlation between the expression of these two markers therefore suggests the possibility that CCR6 may play a role in the development of

aggregates of CD4⁺ T cells that are characteristically found in rheumatoid synovium.

TRANCE is known to be expressed by activated T cells, and can stimulate dendritic cells and osteoclasts [18]. Of note, TRANCE-mediated activation of osteoclasts has recently been shown [19] to play an important role in the damage to bone that is found in experimental models of inflammatory arthritis. It is therefore of interest that TRANCE was expressed by 3–16% of the RA synovial CD4⁺ memory T cells. Of note, 67% of TNF- α -positive cells expressed TRANCE. In concert, TNF- α and TRANCE expressed by this subset of CD4⁺ memory T cells might make them particularly important in mediating the bony erosions that are characteristic of RA.

Interestingly, there was a correlation between expression of IFN- γ and IL-10 in RA peripheral blood CD4⁺ memory T cells. In RA peripheral blood, CD154 expression correlated with that of CXCR3 by CD4⁺ memory T cells. It has been claimed [15] that CXCR3 is preferentially expressed by *in vitro* generated Th1 cells. However, in the present study CXCR3 did not correlate with IFN- γ expression. Although IFN- γ and TNF- α mRNAs were expressed *in vivo* by peripheral blood CD4⁺ T cells from RA patients, LT- α mRNA was not detected, whereas IFN- γ , TNF- α , and LT- α were not detected in samples from healthy donors. These findings indicate that RA peripheral blood CD4⁺ memory T cells are stimulated *in vivo*, although they do not express LT- α mRNA. The present studies indicate that the frequencies of CD4⁺ memory T cells that expressed IFN- γ in the blood and in the synovium are comparable. These results imply that activated CD4⁺ memory T cells migrate between blood and synovium, although the direction of the trafficking is unknown. The presence of LT- α mRNA in synovium, but not in blood, indicates that CD4⁺ memory cells are further activated in the synovium, and that these activated CD4⁺ memory T cells are retained in the synovium until LT- α mRNA decreases.

In conclusion, CD4⁺ memory T cells are biased toward Th1 cells in RA synovium and peripheral blood. In the synovium, IFN- γ and LT- α were produced by individual cells, whereas in the rheumatoid blood no LT- α -producing cells were detected. Furthermore, there were modest correlations between individual cells that expressed particular cytokines, such as IL-10, and certain chemokine receptor mRNAs.

Full article

Introduction

In RA large numbers of CD4⁺ memory T cells infiltrate the inflamed synovium [1–3]. The accumulated CD4⁺ memory T cells in the RA synovium appear to be activated, because they express cytokines and activation markers [4–8]. Expressed cytokines and activation markers should play important roles in the pathogenesis of RA. However, the frequency of cytokine expression by RA synovial CD4⁺ T cells has not been analyzed accu-

rately. Recently, the roles of chemokine and chemokine receptor interactions in T-cell migration have been intensively examined. Interactions of chemokine and chemokine receptors might therefore be important for the accumulation of the T cells in the RA synovium. Accordingly, correlation of cytokine and chemokine receptor expression might be important in delineating the function and potential means of accumulation of individual CD4⁺ memory T cells in the RA synovium.

In order to analyze cytokine, activation marker, and chemokine receptor expression by individual CD4⁺ memory T cells, we used a single-cell RT-PCR technique. The method made it possible to analyze cytokine and chemokine receptor expression by individual RA synovial CD4⁺ memory T cells without *in vitro* stimulation, and to correlate cytokine and chemokine receptor expression.

Using this technique, we analyzed cytokine (IL-2, IL-4, IL-6, IL-10, IL-13, IFN- γ , TNF- α , and LT- α), activation marker (CD154 [CD40 ligand] and TRANCE – also called receptor activator of nuclear factor- κ B ligand [RANKL] or osteoclast differentiation factor [ODF]), and chemokine receptor expression by individual CD4⁺ memory T cells isolated from rheumatoid synovium and blood. The results indicate that CD4⁺ memory T cells are biased toward Th1 cells in RA synovium, although individual cells produced IFN- γ or LT- α , but not both. A similar pattern of cytokine production was observed with CD4⁺ memory T cells from RA blood, with the exception that no cells expressing LT- α were detected. There were modest correlations between individual cells that expressed particular cytokine and chemokine receptor mRNAs.

Materials and method

Specimens

Synovial tissues were obtained at surgery from three RA patients. The synovial tissue was minced and incubated with 0.3 mg/ml collagenase (Sigma, St Louis, MO, USA) for 1 h at 37°C in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA). Partially digested pieces of the tissue were pressed through a metal screen to obtain single-cell suspensions. Mononuclear cells were then isolated by ficoll-hypaque (Pharmacia Biotech, Piscataway, NJ, USA) gradient centrifugation. RA was diagnosed according to the American College of Rheumatology criteria [20].

Also, peripheral blood mononuclear cells were separated by ficoll-hypaque gradient centrifugation from two RA patients and a normal donor.

Single cell sorting and reverse transcription polymerase chain reaction

The method for construction of cDNA libraries from single cells was similar to previously reported techniques [21,22]. RA synovial tissue T cells were analyzed without *in vitro* culture or stimulation by staining synovium mononuclear cells with FITC-conjugated anti-CD4 monoclonal antibody (Q4120; Sigma) and PE-conjugated anti-CD45RO monoclonal antibody (UCHL-1), after which individual CD4⁺CD45RO⁺ T cells were sorted in a 96-well PCR plate (Robbins Scientific, Sunnyvale, CA, USA) using the FACStar^{Plus} (Becton Dickinson, San Jose, CA, USA). Peripheral CD4⁺CD45RO⁺ single cells were also sorted into wells of 96-well PCR plates using the FACStar^{Plus} flow cytometer.

Each well contained 4 μ l lysis buffer (50 mmol/l Tris-HCl [pH 8.3], 75 mmol/l KCl, 3 mmol/l MgCl₂, 1 mmol/l DTT, 10 μ mol/l dNTP [Sigma], 5 U/ml PRIME RNase Inhibitor [5 Prime \rightarrow 3 Prime Incorporated, Boulder, CO, USA], 300 U/ml RNAGuard [Pharmacia Biotech], 200 ng/ml oligo[dT]₂₄ [Integrated DNA Technologies Incorporated, Coralville, IA, USA], and 0.5% NP-40). The samples were heated to 65°C for 1 min, cooled to 20°C for 3 min, and maintained on ice. Two units of AMV Reverse Transcriptase (Promega, Madison, WI, USA) and 50 U of M-MLV Reverse Transcriptase (Life Technologies) was added, and the samples were incubated at 37°C for 15 min before heat inactivation at 65°C for 10 min. For polyadenylate tailing at the 3' end of the cDNA, 5 μ l tailing buffer (200 mmol/l potassium cacodylate [pH 7.2], 4 mmol/l CoCl₂, 0.4 mmol/l DTT), 2 mmol/l dATP (Roche, Indianapolis, IN, USA), and 10 U terminal transferase (Roche) were added, and incubated at 37°C for 20 min, followed by heat inactivation at 65°C for 10 min. To amplify the cDNA non-specifically, PCR was performed with 100 μ l of 10 mmol/l Tris-HCl (pH 9.0), 50 mmol/l KCl, 2.5 mmol/l MgCl₂, 0.01% Triton-X, 1 mmol/l dNTP, 10 U Taq DNA polymerase (Promega), and 2 μ mol/l X-(dT)₂₄ primer (ATG TCG TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC-dT₂₄; Integrated DNA Technologies Incorporated). Twenty-five cycles of amplification were performed with 1 min at 94°C, 2 min at 42°C, and 6 min at 72°C, plus 10 s extension per cycle. Afterward, 5 U Taq DNA polymerase was added, followed by an additional 25 cycles of PCR.

For gene-specific amplification, 1 μ l of nonspecifically amplified cDNA was amplified by PCR in 25 μ l 10 mmol/l Tris-HCl (pH 9.0), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.01% Triton-X, 200 μ mol/l dNTP and 0.625 U Taq DNA polymerase. The cycling program was: 94°C for 1 min, 60°C for 1 min (58°C: IL-2, IL-4, IFN- γ), and 72°C for 1 min for 35 cycles, followed by a final extension for 7 min. For nested amplification, 1 μ l of amplified PCR reaction mixture was added to a second PCR reaction mixture (50 μ l of 10 mmol/l Tris-HCl [pH 9.0], 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.01% Triton-X, 200 μ mol/l dNTP, and 1.25 U Taq DNA polymerase). The cycling program was: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles, followed by a final extension for 7 min. The PCR products were then separated by electrophoresis through 2.0% agarose. The primers were designed to be within 600 bp of the 3' end of each mRNA. The primers used were as shown in Table 3.

To confirm that the PCR products were amplified from the corresponding genes, the nucleotide sequences of the PCR products were analyzed. More than five PCR products of each cytokine from a total of two or three donors were sequenced. All the sequences of the PCR products were identical to the previously published sequences (data not shown).

To confirm that each well contained only one cell after sorting, TCR Vβ mRNA was analyzed by single-cell RT-PCR using Vβ family-specific primers. In the wells analyzed, only one TCR Vβ was detected (data not shown).

Statistical analyses

To analyze correlations between cytokines and chemokine receptor expressions, and to compare frequencies of chemokine receptor-expressing cells between different T-cell subsets, Fisher's exact probability test was used.

Results

Cytokine and activation marker expression by individual rheumatoid synovial tissue CD4+ memory T cells

Cytokine and activation marker expressions by individual CD4+CD45RO+ T cells from RA synovial tissues were analyzed by employing a single-cell RT-PCR method. Expressions of mRNAs were analyzed in 152 individual synovial tissue CD4+CD45RO+ T cells sorted from three RA patients in which TCR Cβ mRNA was detected. Cytokine and activation marker expressions by 50 synovial tissue CD4+CD45RO+ T cells (RA1) are shown in Fig. 1.

Frequencies of CD4+ memory T cells that expressed cytokine and activation marker mRNA in RA synovium are shown in Table 1. IL-2, IL-4, and IL-6 were not expressed by the synovial tissue CD4+CD45RO+ T cells, whereas 2–20% of cells expressed the other mRNAs.

Correlation of the expression of cytokines by rheumatoid synovial tissue CD4+ memory T cells

Few correlations between cytokine and activation marker mRNAs were observed. Notably, no cells contained mRNAs for both IFN-γ and LT-α, cytokines that are thought to define the Th1 phenotype [9]. However, the frequency of TRANCE-positive cells in IL-10-positive cells was significantly higher than that in IL-10-negative cells (Table 2). Moreover, the frequency of TRANCE-positive cells in TNF-α-positive cells was also significantly higher than that in TNF-α-negative cells.

Correlation of cytokine and chemokine receptor expression by RA synovial tissue CD4+ memory T cells

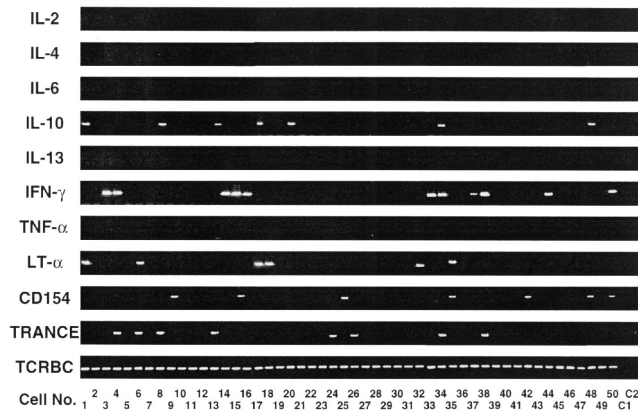
Varying percentages of CD4+ memory T cells expressed CC and CXC chemokine receptors as shown in Tables 4 and 5. Thus, for example, 21% of RA synovial CD4+ memory T cells expressed CCR5, 39% CCRb, and 19% CCR7 mRNAs, whereas 16% expressed CXCR3 and 76% expressed CXCR4 mRNAs. The frequency of CCR5-positive cells in IFN-γ-positive cells was significantly higher than that in IFN-γ-negative cells (Table 4), whereas the frequency of CCR6-positive cells in LT-α-positive cells was significantly higher than that in LT-α-negative cells, and the frequency of CCR7-positive cells in IL-10-positive cells was significantly higher than that in IL-10-negative cells. Furthermore, the frequency of

Table 3

Primers used for PCR amplification

Cytokine/chemokine receptor	Primer
IL-2	5'-TAC AAG AAT CCC AAA CTC ACC AGG A 3'-GTC AGT GTT GAG ATG ATG CTT TGA CA 3' (nested)-TGG TTG CTG TCT CAT CAG CAT ATT CA
IL-4	5'-CTT TGC TGC CTC CAA GAA CAC AAC T 3'-TCT CAT GAT CGT CTT TAG CCT TTC CA 3' (nested)-TCC TTC ACA GGA CAG GAA TTC AAG C
IL-6	5'-GTT CCT GCA GAA AAA GGC AAA G 3'-CTG AGG TGC CCA TGC TAC ATT T 5' (nested)-CTA GAT GCA ATA ACC ACC CCT GA
IL-10	5'-TTA GGA AGA GAA ACC AGG GAG CC 3'-GCT GGC CAC AGC TTT CAA GAA T 5' (nested)-CAG GGA GCC CCT TTG ATG ATT A
IL-13	5'-ACC CAG GGA TGA CAT GTC CCT A 3'-CCC AAC GGT GAC AAA CAC ACT C 3' (nested)-AAG AAG TGT GGC TGA GCT GCT GT
IFN-γ	5'-AAG GCT TTA TCT CAG GGG CCA ACT 3'-TGG AAG CAC CAG GCA TGA AAT CTC 5' (nested)-CAA GAT CCC ATG GGT TGT GTG TTT
TNF-α	5'-GGC TCA GAC ATG TTT TCC GTG A 3'-CTC AGC AAT GAG TGA CAG TTG G 5' (nested)-CAA TAG GCT GTT CCC ATG TAG CC
LT-α	5'-CCT GAA CCA TCC CTG ATG TCT G 3'-AAA TAG TCC CCT CCC TGC CTC T 3' (nested)-TCT AGT CAT CCC CCA AGC TCC TC
CD154	5'-AGT CAG GCC GTT GCT AGT CAG T 3'-GGA AAC AAT GGA GAC TGC AGG TA 3' (nested)-TAA TGA GGA GTG GGC AGG CTC AG
TRANCE	5'-GAA AAC TTG CAG CTA AGG AGG GG 3'-GGT GGC CAA CAT CCT GCT TAT T 3' (nested)-CCT GCT TAT TAT TCA AGG CAT CC
CCR1	5'-GAT TTG GGC TCT TGG AAT CCT G 3'-GTG CTT AGC CCA CTC CCT GAA T 3' (nested)-AGG GCT TTC TTA GTT CCA CTG CC
CCR2	5'-TGG GAG TTT TGG TGG AGT CCG AT 3'-GGG GGA TGT GGC CTA AGA AGC AT 3' (nested)-GCC TAA GAA GCA TCT GAA CAA TGG
CCR3	5'-CTA AGG TCA TTA CCA CAG GCC AGG 3'-AGC AGG GAA AGA ACT AGG CAC ATT 5' (nested)-GCA GCG TAC TCA TCA TCA ACC C
CCR5	5'-CTC AGG GAA TGA AGG TGT CAG A 3'-TGC TAC TGT TGC ACT CTC CAC AAC T 5' (nested)-AGC CTC TGA ATA TGA ACG GTG AGC
CCR6	5'-CAT GGA ACT CAT GTT TTT AAA GGG C 3'-CCA TGC CTA GCC CAT GAC AGT A 3' (nested)-CCC ATG ACA GTA CCT TCC TAA CA
CCR7	5'-AGC ACA CTC ATC CCC TCA CTT G 3'-AGC CAA GAG CTG AGT GCA TGT C 3' (nested)-GAG TGC ATG TCA TCC CCA CTC T
CXCR1	5'-CAG ATC TAT GCC ACA AGA ACC CC 3'-CTT TCT AGG GAT GCT GAT GCT CC 3' (nested)-CTG ATG CTG CAC GCC AGC CTG GA
CXCR2	5'-ATT ACC AGG GAC TGA GGG GAG GG 3'-GTG GCA TTA AGT CAC ATT GCG G 3' (nested)-GTC ACA TTG CGG TAC AAC TAT CAC
CXCR3	5'-CAC TGC CCT TCT CAT TTG GAA ACT 3'-GCA AAT ATA GAG GTC TTG GGG AC 5' (nested)-AGT ACA AGG CAT GGC GTA GAG GG
CXCR4	5'-GGA CCT GTG GCC AAG TTC TTA GTT 3'-ACT GTA GGT GCT GAA ATC AAC CCA 3' (nested)-CAG CTG GGG ATC ATT TCT AGC TTT
CXCR5	5'-CAG GAC AAC GAG AAA GCC CTA AG 3'-GGT CTC TGT GCT GCC TGT ACT GT 5' (nested)-GTA TCT CCT CGC AAG CTG GGT AA
TCR Cβ	5'-TCA AGT CCA GTT CTA CGG GCT C 3'-TCA TAG AGG ATG GTG GCA GAC A 5' (nested)-CTC TCG GAG AAT GAC GAG TGG AC

Figure 1



Cytokine and activation marker expressions by CD4⁺CD45RO⁺ T cells from RA synovial tissue (RA1). RA synovial tissue CD4⁺CD45RO⁺ T cells were sorted, and cytokine and activation marker expressions were immediately determined in the TCR Cβ (TCRBC) mRNA-positive wells employing single-cell RT-PCR. Amplified PCR products were separated by electrophoresis through 2.0% agarose. As negative controls, one well that did not contain a cell (C1) and one well that was not prepared with reverse transcriptase (C2) were analyzed.

CXCR4-positive cells in TRANCE-positive cells was significantly higher than that in TRANCE-negative cells (Table 5).

Cytokine and activation marker expression by peripheral blood CD4⁺ memory T cells from RA patients and a normal donor

Expressions of cytokine and activation marker mRNAs was also analyzed in 48 individual peripheral blood CD4⁺CD45RO⁺ T cells sorted from two RA patients and in 33 individual peripheral blood CD4⁺CD45RO⁺ T cells sorted from a normal donor. Frequencies of CD4⁺ memory T cells that expressed cytokine and activation marker mRNA are shown in Table 6. IL-2, IL-4, IL-6, and LT-α were not expressed by the RA peripheral blood CD4⁺CD45RO⁺ T cells, whereas 4–17% of cells expressed the other markers. The most striking difference between RA synovial tissue and peripheral blood CD4⁺ memory T cells was the presence of LT-α expression in the former, but not in the latter. IFN-γ and TNF-α were not expressed by normal peripheral blood CD4⁺ memory T cells, although they were expressed by RA peripheral blood CD4⁺ memory T cells.

Variable frequencies of RA peripheral CD4⁺ memory T cells expressed chemokine receptor mRNAs. Except for significantly decreased expressions of CCR5 and CXCR4, there were no differences between chemokine receptor expressions by synovial tissue and peripheral blood CD4⁺ memory T cells. In peripheral blood CD4⁺ memory T cells, there was a significant correlation between IFN-γ and IL-10 expressions, and IFN-γ and CCR6 expressions (*P* < 0.05; Table 7). In addition, CD154 and CXCR3 expressions correlated (*P* < 0.005). No other correlations were detected (data not shown).

Table 4

Correlation of cytokine and CC chemokine receptor expression in 152 individual RA synovial tissue CD4⁺ memory T cells

	CCR1	CCR2	CCR3	CCR5	CCR6	CCR7	Total
IL-10 (+)	2 (13)	1 (6)	2 (13)	4 (25)	8 (50)	7 (44)*	16
IL-10 (-)	15 (11)	7 (5)	5 (4)	28 (21)	51 (38)	22 (16)	136
IL-13 (+)	0 (0)	0 (0)	0 (0)	1 (20)	3 (60)	2 (40)	5
IL-13 (-)	17 (12)	8 (5)	7 (5)	31 (21)	56 (38)	27 (18)	147
IFN-γ (+)	0 (0)	3 (17)	1 (6)	8 (44)*	9 (50)	4 (22)	18
IFN-γ (-)	17 (13)	5 (4)	6 (4)	24 (18)	50 (37)	25 (19)	134
TNF-α (+)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3
TNF-α (-)	17 (11)	8 (5)	7 (5)	32 (21)	59 (40)	29 (19)	149
LT-α (+)	0 (0)	1 (9)	0 (0)	1 (9)	8 (73)*	4 (36)	11
LT-α (-)	17 (12)	7 (5)	7 (5)	31 (22)	51 (46)	25 (18)	141
CD154 (+)	3 (10)	4 (13)	1 (3)	6 (19)	14 (45)	9 (29)	31
CD154 (-)	14 (12)	4 (4)	6 (5)	26 (21)	45 (37)	20 (17)	121
TRANCE (+)	1 (8)	1 (8)	2 (17)	3 (25)	6 (50)	4 (33)	12
TRANCE (-)	16 (11)	7 (5)	5 (4)	29 (21)	53 (38)	25 (18)	140

Values are expressed as number (percentage) of cytokine-positive cells. **P* < 0.05.

Table 5

Correlation of cytokine and CXC chemokine receptor expression in 152 individual RA synovial tissue CD4⁺ memory T cells

	CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	Total
IL-10 (+)	1 (7)	1 (7)	2 (13)	13 (81)	2 (13)	16
IL-10 (-)	9 (7)	12 (9)	23 (17)	103 (76)	14 (10)	136
IL-13 (+)	0 (0)	0 (0)	1 (20)	5 (100)	0 (0)	5
IL-13 (-)	10 (7)	13 (9)	24 (16)	111 (76)	16 (11)	147
IFN-γ (+)	3 (17)	2 (11)	4 (22)	15 (83)	2 (11)	18
IFN-γ (-)	7 (5)	11 (8)	21 (16)	101 (75)	14 (10)	134
TNF-α (+)	0 (0)	0 (0)	0 (0)	3 (100)	0 (0)	3
TNF-α (-)	10 (7)	13 (9)	25 (17)	113 (76)	16 (11)	149
LT-α (+)	1 (9)	0 (0)	2 (18)	10 (91)	2 (18)	11
LT-α (-)	9 (6)	13 (9)	23 (16)	106 (75)	14 (10)	141
CD154 (+)	3 (10)	2 (6)	5 (16)	27 (87)	6 (19)	31
CD154 (-)	7 (6)	11 (9)	20 (17)	89 (74)	10 (8)	121
TRANCE (+)	1 (8)	1 (8)	2 (17)	12 (100)*	2 (17)	12
TRANCE (-)	9 (6)	12 (9)	23 (16)	104 (74)	14 (10)	140

Values are expressed as number (percentage) of cytokine-positive cells. **P* < 0.05.

Table 6

Frequencies of cytokine and activation marker expression by CD4⁺CD45RO⁺ T cells in peripheral blood of RA patients and a normal donor

	IL-2	IL-4	IL-6	IL-10	IL-13	IFN-γ	TNF-α	LT-α	CD154	TRANCE
RA										
RA3 (n = 24)	0 (0)	0 (0)	0 (0)	3 (13)	0 (0)	3 (13)	2 (8)	0 (0)	4 (17)	3 (13)
RA4 (n = 24)	0 (0)	0 (0)	0 (0)	3 (13)	2 (8)	2 (8)	2 (8)	0 (0)	4 (17)	2 (8)
Total (n = 48)	0 (0)	0 (0)	0 (0)	6 (13)	2 (4)	5 (10)	4 (8)	0 (0)	8 (17)	5 (10)
Normal donor										
Donor 1 (n = 33)	0 (0)	0 (0)	0 (0)	5 (15)	1 (3)	0 (0)	0 (0)	0 (0)	2 (6)	2 (6)

Values are expressed as number (percentage) of cytokine-positive cells in TCR Cβ-positive cells.

Table 7

Correlation of cytokine and chemokine receptor expression in individual CD4⁺ memory T cells of RA peripheral blood

	IFN-γ	CD154
IL-10 (+)	3/3 (100)*	
IL-10 (-)	2/45 (4)	
CCR6 (+)	4/13 (31)*	
CCR6 (-)	1/35 (3)	
CXCR3 (+)		4/6 (67) [†]
CXCR3 (-)		4/42 (10)

Values are expressed as IFN-γ or CD154 positive cells/cytokine or chemokine receptor positive or negative cells (percentage). **P* < 0.05, [†]*P* < 0.005.

Discussion

The present study employed single-cell PCR technology to analyze cytokine mRNA expressions by unstimulated RA synovial tissue CD4⁺ memory T cells immediately after isolation, without *in vitro* manipulation. The results are consistent with the Th1 nature of rheumatoid inflammation. These data showed that 6–22% of RA synovial CD4⁺ memory T cells produced IFN-γ mRNA. Previous studies [4–6] reported that 1–10% of RA synovial T cells expressed IFN-γ protein by immunohistologic analysis. Although there is some variation in the results obtained with the different methodologies, both results are consistent with the conclusion that there is a Th1 bias in RA [3,23]. It is noteworthy that no individual synovial CD4⁺ memory T cells expressed both IFN-γ and LT-α mRNA,

even though these are the prototypic Th1 cytokines [9]. These results imply that, in the synovium, regulation of IFN- γ and LT- α must vary in individual cells, even though both Th1 cytokine mRNAs can be expressed.

The present data showed that CCR5 expression correlated with IFN- γ but not with LT- α expression by synovial CD4⁺ memory T cells. It has been reported that CCR5 expression is upregulated in RA synovial fluid and synovial tissue T cells [10–12], and that CCR5 Δ 32 deletion may have an influence on clinical manifestations of RA [13], suggesting that CCR5 might play an important role in RA. Recently, it has been claimed [14,15] that CCR5 was preferentially expressed by Th1 cell lines. In the present study, however, CCR5 was not expressed by all IFN- γ -expressing cells. Moreover, CCR5 expression did not correlate with expression of LT- α by RA synovial CD4⁺ memory T cells, although it correlated with IFN- γ . Therefore, it is unclear whether CCR5 is a marker of Th1 cells in RA synovium.

Of RA synovial CD4⁺ T cells 6–14% expressed IL-10, and the expression correlated with CCR7 expression. It has been reported that approximately 1.5% of synovial T cells express IL-10 by immunohistochemistry [5], and that 4% of synovial CD4⁺ T cells have the potential to express IL-10 [3]. Recently, it was reported [16] that, in the blood, CCR7⁺CD4⁺ memory T cells express lymph-node homing receptors and lack immediate effector function, but efficiently stimulate dendritic cells. However, because 19% of RA tissue CD4⁺ memory T cells expressed CCR7 and there was a correlation between IL-10 production and CCR7 expression, these cells may play a unique role in the synovium as opposed to in the blood. By producing IL-10, they may exert an immunoregulatory function. In addition, it is interesting to note that IL-10 expression also correlated with expression of TRANCE. Although it is possible that IL-10 produced by these cells inhibited T-cell activation in the synovium, TRANCE expressed by these same cells might function to activate dendritic cells and indirectly stimulate T cells, mediating inflammation in the synovium. These results imply that individual T cells in the synovium might have different, and sometimes opposite functional activities.

LT- α was expressed by 3–12% of the synovial CD4⁺ memory T cells, and the expression correlated with CCR6 expression, which is expressed by 39% of the synovial CD4⁺ memory T cells. However, there were no LT- α -expressing CD4⁺ T cells that also produced IFN- γ , although synovial CD4⁺ memory T cells that produced each cytokine were found in abundance. It has been reported that CCR6 is expressed by resting peripheral memory T cells [17], whereas LT- α expression is associated with the presence of lymphocytic aggregates in synovial tissue [7]. The correlation between the expression of

these two markers therefore suggests the possibility that CCR6 might play a role in the development of aggregates of CD4⁺ T cells that are characteristically found in rheumatoid synovium.

TRANCE is known to be expressed by activated T cells, and can stimulate dendritic cells and osteoclasts [18]. Of note, TRANCE-mediated activation of osteoclasts has recently been shown [19] to play an important role in the damage to bone found in experimental models of inflammatory arthritis. Recently, the presence of TRANCE in rheumatoid synovium was reported [24,25]. It is therefore of interest that TRANCE was expressed by 3–16% of the RA synovial CD4⁺ memory T cells. Of note, TRANCE expression correlated with IL-10, TNF- α , and CXCR4 expressions. Especially noteworthy was that 67% of TNF- α -positive cells expressed TRANCE. In concert, TNF- α and TRANCE expressed by this subset of CD4⁺ memory T cells might make them particularly important in mediating the bony erosions that are characteristic of RA.

Interestingly, there was a correlation between expression of IFN- γ and IL-10 in RA peripheral blood CD4⁺ memory T cells. Production of IL-10 in humans differs from that in the mouse, in that IL-10 production does not appear to be restricted to Th2 cells [9]. As noted here, RA peripheral CD4⁺ T cells could express both IFN- γ and IL-10. In RA peripheral blood, CD154 expression correlated with CXCR3 by CD4⁺ memory T cells. It has been claimed [15] that CXCR3 was preferentially expressed by *in vitro* generated Th1 cells. However, in the present study CXCR3 did not correlate with IFN- γ expression. Although IFN- γ and TNF- α mRNAs were expressed *in vivo* by peripheral blood CD4⁺ T cells from RA patients, LT- α mRNA was not detected, whereas IFN- γ , TNF- α , and LT- α were not detected from healthy donors. These findings indicate that RA peripheral blood CD4⁺ memory T cells are stimulated *in vivo*, but that they do not express LT- α mRNA. Previous studies have documented the presence of IFN- γ and IL-10 mRNA in circulating T cells of RA patients [26,27]. The present studies indicate that the frequency of CD4⁺ memory T cells that express IFN- γ in the blood and synovium is comparable, although the percentages that secrete IFN- γ are not known. These results imply that activated CD4⁺ memory T cells migrate between blood and synovium, although the direction of the trafficking is unknown. The presence of LT- α mRNA in synovium but not in blood indicates that CD4⁺ memory cells are further activated in the synovium, and that these activated CD4⁺ memory T cells are retained in the synovium until LT- α mRNA decreases.

In conclusion, CD4⁺ memory T cells are biased toward Th1 cells in RA synovium and peripheral blood. In the synovium, IFN- γ and LT- α were produced by individual cells, whereas in the rheumatoid blood no LT- α -producing cells

were detected. Furthermore, there were modest correlations between individual cells that expressed particular cytokines and certain chemokine receptor mRNAs.

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