T cell differentiation in autoimmune diseases
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Citation for published version (APA):

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Chapter 2

Quantitation of interferon gamma and interleukin-4-producing T cells in synovial fluid and peripheral blood of arthritis patients

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Rheumatology 38:214–220.1999
Quantitation of interferon gamma- and interleukin-4-producing T cells in synovial fluid and peripheral blood of arthritis patients

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Abstract

Objective. The balance between T cells able to produce interferon gamma (IFN-\textgamma) (type 1) and interleukin-4 (IL-4) (type 2) is considered to be important in the development of autoimmunity. In this study, we quantitated the percentage of both cell types in synovial fluid (SF) and peripheral blood (PB) of rheumatoid arthritis (RA) patients, non-rheumatoid arthritis patients and healthy controls.

Methods. After short-term stimulation of synovial mononuclear cells with phorbol ester and ionomycin, cytokine-producing cells were quantitated using an intracellular staining technique and flow cytometric analysis.

Results. Although no significant differences in CD8\textsuperscript{+} cells were found, significantly higher percentages of IFN-\textgamma-producing CD4\textsuperscript{+} (Th1) and IL-4-producing CD4\textsuperscript{+} (Th2) cells were found in the peripheral blood of RA patients in comparison with healthy controls. However, the Th1/Th2 ratio was not different between the two groups. Comparative studies between PB and SF showed that in both RA and non-RA patients, percentages of Th1 cells were higher in SF than in PB, while Th2 cells were preferentially found in the PB, resulting in a higher Th1/Th2 ratio in the SF. The Th1/Th2 ratio in the SF correlated with disease activity as estimated by the erythrocyte sedimentation rate.

Conclusion. These results are in agreement with the hypothesis that Th1 cells preferentially home to inflamed joints in both RA and non-RA patients, but show that this does not result in an altered Th1/Th2 ratio in the PB of RA patients.

Key words: Rheumatoid arthritis, Th1/Th2 cells, Cytokines.

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting primarily synovial tissue in multiple joints. A marked hyperplasia of synoviocytes and blood vessels in the synovium, and a mononuclear cellular infiltrate consisting of macrophages, T and B cells, are found. There is controversy on the issue of which cell is crucial in the inflammatory process [1, 2]. Nevertheless, CD4\textsuperscript{+} T-helper cells (Th) appear to play an important role, since susceptibility to RA is specifically associated with class II MHC alleles possessing a shared epitope [3]. CD4\textsuperscript{+} T cells can differentiate into two distinct subsets designated Th1 and Th2 type cells, which are characterized by different cytokine production profiles and effector functions. Th1 cells produce interleukin-2 (IL-2) and interferon gamma (IFN-\textgamma), support macrophage activation and are involved in delayed-type hypersensitivity responses. Th2 cells, on the other hand, secrete IL-4, IL-5 and IL-13, and provide efficient help for B-cell activation, antibody production and down-modulate the production of pro-inflammatory cytokines by macrophages. From animal experiments, it has become clear that balances between Th1 and Th2 cells, or their cytokines, are important in the induction or prevention of organ-specific autoimmune disease [4]. Several reports have been published on the detection of Th1 and Th2 cytokines in RA [5-13]. Using either immunohistochemistry or ELISPOT techniques, evidence was obtained for an increased IFN-\textgamma/IL-4 ratio in synovial fluid (SF) compared to peripheral blood (PB).
To obtain information on the phenotype of the cytokine-producing T cells in this study, three-colour immunofluorescence analysis with intracellular staining was used to quantify cytokine-producing T cells in PB and SF of arthritis patients. Our findings show that in both arthritis patients and age-matched healthy controls, IFN-γ- and IL-4-secreting T cells are contained within the memory (defined as CD95+ ) compartment. Furthermore, comparison between PB and SF showed that higher percentages of IFN-γ-secreting, but not of IL-4-producing cells, are found in the joint, resulting in a relatively high local Th1/Th2 ratio.

Materials and methods

Isolation of mononuclear cells

SF, PB, or both, were obtained in heparinized tubes. SF and PB were diluted in phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS) and 5% trisodium citrate. Mononuclear cells were isolated by density centrifugation on a Ficoll-Hypaque gradient (d = 1.079). Cells were suspended in Iscove’s modified Dulbecco’s medium (IMDM) containing 30% FCS and 10% dimethylsulphoxide, and stored in liquid nitrogen until use. Peripheral blood mononuclear cells (PBMC) of a healthy donor were processed in the same way and used as standard control cells.

Immunofluorescence

T cells producing IFN-γ and IL-4 were detected by intracellular staining with flow cytometric analysis as described before [14-16]. Briefly, 1.2 million thawed viable mononuclear cells were stimulated in 1 ml IMDM/10% FCS with 1 ng phorbol myristate acetate (PMA; Sigma Chemicals) and 1 μM ionomycin (Sigma Chemicals). Monensin (1 μM; Sigma Chemicals) was added to prevent excretion of the newly synthesized cytokines. After 4 h, cells were washed in PBS/0.5% bovine serum albumin (BSA) and stained with directly labelled monoclonal antibodies (mAb) against surface molecules for 20 min. After two washing steps with PBS/BSA and one with PBS, cells were fixed with 4% paraformaldehyde during 10 min. Fixation was stopped by washing the cells three times in PBS. Cells were permeabilized with 0.1% saponin in PBS/BSA supplemented with 10% pooled human serum (HPS) to block aspecific binding of mAb. Phycoerythrin- or biotin-labelled mAb directed against IFN-γ or IL-4, and an irrelevant IgG1 murine mAb as a negative control, were diluted in PBS/BSA/saponin to a concentration of 5 μg/ml and added to the cells in aliquots of 50 μl for 25 min at 4°C. After binding of streptavidin-RED670 to the biotinylated antibodies, cells were analysed on a FACSscan (Becton Dickinson). IFN-γ- and IL-4-producing T cells were identified by setting a marker on the sample containing the negative control antibody (Fig. 1a). To correct for daily variation, standard control PBMC of one healthy donor were used. The relative amounts of cytokine-producing cells in the PB and SF were defined as the percentage positive cells measured in study samples divided by that of the standard control PBMC. By way of this normalization procedure, results of individual patients analysed in different sets of experiments could be compared. In experiments that compared results from SFM C and PBMC from the same patient, percentages of CD8− and CD8+ cytokine-positive cells were first divided by the percentage CD95+ cells. Percentages of CD95 + CD8+ and CD95 + CD8+ cells were determined by flow cytometric analysis after staining of mononuclear cells with directly labelled antibodies.

Antibodies

Phycoerythrin (PE)-anti-CD3, CD4, CD8, IFN-γ, IL-4, fluorescein isothiocyanate (FITC)-anti-CD8 and PerCP-anti-CD4 were purchased from Becton Dickinson. Biotinylated anti-IFN-γ (MD1, IgG1) was obtained from Dr P. van der Meide, TNO, Rijswijk, The Netherlands. Biotinylated anti-IL-4 (5A4, IgG1) was kindly provided by Dr T. van der Pouw-Kraan, CLB, Amsterdam, The Netherlands. Binding of biotinylated antibodies was visualized by binding of streptavidin-coupled REDI670 (Dako, Denmark). Anti-CD4-PE cyanin 5.1 and anti-CD95-FITC were purchased from

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![Figure 1](https://example.com/f1.png)

**Fig. 1.** Flow cytometric analysis after simultaneous staining with anti-CD95 and negative control antibody (a), IFN-γ (b) or IL-4 (c) of CD4+ gated PBMC of an RA patient.
Table 1. Percentage of CD95 + cells within different T-cell subsets in the peripheral blood of RA patients and healthy controls. The medians are given with the range in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>RA (n = 23)</th>
<th>Controls (n = 23)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 +</td>
<td>54.2</td>
<td>48.1</td>
<td>0.02</td>
</tr>
<tr>
<td>CD8 +</td>
<td>62.2</td>
<td>55.9</td>
<td>0.27</td>
</tr>
<tr>
<td>CD4 + IFN-γ+</td>
<td>91.4</td>
<td>83.4</td>
<td>0.15</td>
</tr>
<tr>
<td>CD4 + IL-4+</td>
<td>95.0</td>
<td>92.9</td>
<td>0.05</td>
</tr>
<tr>
<td>CD8 + IFN-γ+</td>
<td>80.4</td>
<td>73.7</td>
<td>0.100</td>
</tr>
<tr>
<td>CD8 + IL-4+</td>
<td>83.3</td>
<td>81.4</td>
<td>0.98</td>
</tr>
<tr>
<td>CD8 + IL-4+</td>
<td>94.2</td>
<td>95.2</td>
<td>0.75</td>
</tr>
</tbody>
</table>

significance. Within the CD4 + subset, the percentages of both IFN-γ- and IL-4-producing cells were significantly higher in the RA patients in comparison with the healthy controls (Fig. 2a and b). However, within the cytokine-enriched CD4 + CD95 + subset, no significant difference was found (Fig. 2d and e). The IFN-γ+:IL-4 ratios within the CD4 + or in the CD4 + CD95 + subset (Th1:Th2 ratio) were not different between patients and controls (Fig. 2c and f). Within the CD8 + subset, neither the relative percentages of IFN-γ- and IL-4-producing cells nor their ratio differed between patients and controls.

Comparison of IFN-γ- and IL-4-producing cells between SF and PB in arthritis patients

To analyse the relationship between IFN-γ- and IL-4-producing T cells in the PB and SF, both cell types were determined in paired samples from 12 patients (seven RA, five non-RA). Since the production of IFN-γ- and IL-4 by T cells is highly enriched in the CD95 + subset (Fig. 1 and Table 1), differences in percentages of CD95 + cells between SF and PB affect the comparison of IFN-γ- and IL-4-producing T cells between these two compartments. Therefore, IFN-γ- and IL-4-producing cells were expressed as percentage of CD95 + cells determined by three-colour flow cytometry. Again, results were expressed as relative amounts compared with PBMC from a healthy control. Both in CD8 + and CD8 + cells, IFN-γ- and IL-4-producing cells were found in higher percentages in the SF, while CD8:IL-4-positive cells were overexpressed in the PB (Fig. 3a, b and d). Therefore, a higher Th1:Th2 ratio was found in the SF (Fig. 3c). This finding was consistent in both RA and non-RA patients. Comparison of the values for cytokine-producing cells between SF and PB yielded a significant correlation for Th1 cells, but not for Th2 cells or the Th1:Th2 ratio (Table 2).

Correlation between IFN-γ- and IL-4-producing T cells in SF and clinical parameters

IFN-γ- and IL-4-positive CD8 + T cells were determined in SF samples of 30 arthritis patients (16 RA and 14 non-RA). Correlations were calculated between the relative percentages of cytokine producers and clinical parameters of all arthritis patients. No significant correlation could be found between age, disease duration, or cytokine producers. However, a significant inverse correlation was observed between the erythrocyte sedimentation rate (ESR) and IL-4 producers in the SF, which resulted in a positive correlation between the ESR and Th1:Th2 ratio as well (Table 2).

Discussion

The role of T cells in RA is still controversial. Several attempts have been made to detect Th1 and Th2 cytokines or their encoding mRNAs in blood [8, 12, 13], synovium [5–7, 9, 11, 18] or SF [7, 9, 10, 12] of RA patients and, depending on the methodology used, both Th1 and Th2 dominance have been reported. Two
Significance between the patient groups was calculated by a non-parametric test (Mann–Whitney).
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**Table 2.** Spearman rank correlation coefficients between cytokine producers in SF, PB and clinical data of both RA and non-RA patients

<table>
<thead>
<tr>
<th></th>
<th>SFMC IFN+CD4+</th>
<th>SFMC IL4+CD4+</th>
<th>SFMC Th1/Th2</th>
<th>PBMC IFN+CD4+</th>
<th>PBMC IL4+CD4+</th>
<th>PBMC Th1/Th2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.11</td>
<td>-0.54</td>
<td>0.25</td>
<td>-0.15</td>
<td>-0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>Disease duration</td>
<td>-0.33</td>
<td>-0.26</td>
<td>-0.10</td>
<td>-0.11</td>
<td>0.58</td>
<td>0.10</td>
</tr>
<tr>
<td>ESR</td>
<td>-0.26</td>
<td>-0.56**</td>
<td>0.48*</td>
<td>0.25</td>
<td>-0.07</td>
<td>0.63*</td>
</tr>
<tr>
<td>SFMC IFN+CD4+</td>
<td></td>
<td></td>
<td></td>
<td>0.66*</td>
<td>0.10</td>
<td>0.64*</td>
</tr>
<tr>
<td>SFMC IL4+CD4+</td>
<td></td>
<td></td>
<td></td>
<td>0.31</td>
<td>0.34</td>
<td>0.63*</td>
</tr>
<tr>
<td>SFMC Th1/Th2</td>
<td></td>
<td></td>
<td></td>
<td>0.30</td>
<td>0.55</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01.

reports have been published on the paired quantitation of IFN-γ- and IL-4-producing cells in PB and SF of RA patients. Significant increases in IFN-γ-producing cells in the SF [19, 20], a significant increase in IL-4-producing cells in unstimulated cells from the SF [20] and no difference in IL-4 producers in stimulated cells [19, 20] were found. Importantly, in previous studies, no attempts were made to correlate cytokine-producing capacity with membrane phenotype. Nevertheless, this is likely to be relevant since the T cells present in the rheumatoid SF are largely CD4+CD45RO+CD95+ 'memory' cells, while in the PB both naive (CD45RO−CD95−) and memory cells are found [21]. In order to differentiate between naive and memory type cells, we used an intracellular cytokine staining technique combined with cell surface phenotype analysis. This flow cytometric analysis was first described by Jung et al. [22] and has since then been modified and used for different purposes [14–16, 23–25]. After short (4 h) stimulation with PMA and ionomycin, both types of cells could be detected in CD4+, CD3+CD8− and CD8+ cells of every individual patient. We found that both IFN-γ- and IL-4-producing CD4+ cells were largely CD95+ in PB of RA patients and healthy controls (Table 1); moreover, >95% of all SF CD4+ cells were CD95+ (data not shown).

In all PBMC and SFMC of arthritis patients tested, more IFN-γ- than IL-4-producing T cells were found, which was not different from results with PBMC of healthy donors. However, significantly more IFN-γ- and IL-4-producing CD4+ T cells were found in RA patients in comparison with healthy controls. Since the percentage of CD95+ cells within the CD4+ populations is higher in RA patients (Table 1), cytokine producers were also determined within the CD95+ subset. No significant difference could be seen in the percentages of IFN-γ- or IL-4-producing cells within the CD4+CD95+ subset. Therefore, it can be concluded that part of the higher percentages of cytokine-producing CD4+ cells in the PB of RA patients can be ascribed to higher percentages of CD95+ cells. We could not find a difference in the IFN-γ/IL-4 ratio either within the CD4+ or in the CD8+ subsets when patients and healthy controls were compared. These findings are in agreement with those of Ronnelid et al. [20] who used an ELISPOT technique without any in vitro stimulation and did not find a difference in the IFN-γ/IL-4 ratio of total PBMC. Van Roon et al. [26], using an in vitro culture system, reported higher IFN-γ/IL-4 ratios in RA compared to controls. Differences in the various studies may possibly be explained by the fact that the latter study depends on long-term in vitro culture.

In RA patients, IFN-γ-producing T cells are reported to be found in higher percentages in the SF than in PB [12, 19]. This study shows that when only the subset of PB T cells is considered that is present in the SF as well (CD95+), this finding can be supported for both RA and non-RA patients, and shows in addition that there is an even more striking difference in IL-4-positive CD8− T cells (considered CD4+) between SF and PB. In all patients tested, lower percentages were found in the SF, resulting in significantly higher Th1/Th2 ratios.
in the SF. This finding may be largely due to different homing properties of Th1 and Th2 cells since murine Th1 cells are shown to home better to inflamed synovial tissue than Th2 cells [27]. In addition, we have shown that human Th1 cells express higher levels of CD94d (Maurice et al., submitted), which is used in the binding to activated endothelium as well as adhesion to fibronectin, which is abundantly expressed in the rheumatoid synovium [28]. Remarkably, the percentage of Th1 cells in the SF correlates with that in the PB, while no correlation between those two compartments could be found for Th2 cells. This might point to the possibility that, apart from different homing properties, Th2 cells are modulated in the synovium while Th1 cells are not. It could very well be that Th2 cells entering the synovium are downregulated by the pro-inflammatory cytokines present since IL-1 is known to inhibit IL-4 expression [29, 30]. This would explain the inverse correlation observed between ESR and Th2 cells in the SF since pro-inflammatory cytokines are major contributors to elevation of the ESR. Alternatively, it could be hypothesized that in patients with low numbers of Th2 cells within the synovium, Th1 cells can amplify the release of macrophage-derived pro-inflammatory cytokines, which results in a higher ESR. The results of this study are in agreement with the hypothesis that Th1 cells preferentially home to inflamed joints in both RA and non-RA patients, but show that this does not result in an altered Th1/Th2 ratio in the PB of RA patients.

Acknowledgement

This study was supported by the Dutch League Against Rheumatism.

References


