T cell differentiation in autoimmune diseases
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Chapter 6

T cell modifying effects of disease modifying anti-rheumatic drugs in arthritis patients

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Abstract

OBJECTIVE: Disease modifying anti-rheumatic drugs (DMARDs) are widely used in the treatment of arthritis. Although DMARDs have been reported to suppress T cell function in vitro their mode of action in vivo is still unclear. Synergy between individual drugs does however suggest the existence of several mechanisms. To examine whether DMARDs have comparable effects on T cells in vivo, we analyzed the phenotype and function of peripheral blood T cells in patients with early arthritis treated with hydroxychloroquine (HCQ), sulfasalazine (SASP) or methotrexate (MTX).

METHODS: The following parameters were evaluated in peripheral blood T cells: 1. Differentiation state as determined by expression of CD45RA, CD27, Beta-1-, Beta-2 integrins, CCR5, CXCR-3 and CCR3. 2. Cytokine profile as determined by intracellular staining for IFN-γ and IL-4 with flowcytometric analyses and 3. Proliferative capacity was determined by thymidine incorporation after CD2 and CD28 stimulation in vitro. A comparison was made just before and 3 months after the start of a new DMARD.

RESULTS: After treatment for three months all groups improved clinically, reflected by a significant decrease in the disease activity score. In SASP treated patients the percentages of IFN-γ positive CD4+ and CD8+ cells decreased significantly in contrast to IL-4 positive cells that were only affected in CD4+ cells. No significant changes in T cell phenotype as determined by the expression of the differentiation markers CD45RA and CD27 were observed during this treatment. In addition a concomitant reduction in the adhesion molecules CD29, CD49d, CD49e and CD18 was observed on T cells in SASP treated patients. We could not find any cytokine reducing effect in HCQ or MTX treated patients nor was there any difference in expression of adhesion molecules on T cells. MTX treated patients showed a reduction of the chemokine receptor CCR5 while HCQ treated patients showed reduced T cell proliferative responses to the combination anti-CD2 and anti-CD28 antibodies.

CONCLUSION: These results indicate that in vivo DMARDs have distinct T cell modulating effects, which could be an explanation for their synergistic effect in the treatment of arthritis.

Introduction

The use of disease modifying anti-rheumatic drugs (DMARDs) is essential in the treatment of chronic arthritis. Despite DMARDs can diminish the amount of inflammation in the joints, their effect is only partial. In rheumatoid arthritis (RA) the combination of DMARDs is more effective than monotherapy. Since there is still uncertainty about both the key mechanisms in the perpetuation of arthritis as well as the major mode of action of DMARDs in vivo, the background for synergy of DMARDs is unclear. T lymphocytes are a major component of the synovial infiltrate in many inflammatory joint diseases. The contribution of these cells to the inflammatory process can be assumed on the observation that they are highly enriched for the proinflammatory type-1 (IFN-γ
producing) cells and lack the anti-inflammatory type-2 (IL-4 producing) subset. The anti-inflammatory effect of DMARDs could be ascribed in part by affecting T cells, since specific effects like inhibition of T cell proliferation and cytokine production by T cells have been documented in vitro. Additional mechanisms to interfere with T cells could be modulating their expression of molecules used for extravasation and adherence to the synovium (homing). This is a multistep procedure in which selectins, integrins and chemokine receptors are involved. Little research has been performed on the effect of DMARDs on any of those participating molecules expressed by T cells. Special attention has been made to the chemokine receptors CXCR3 and CCR5. They seem to play an important role in lymphocyte homing to inflamed tissue since the majority of the T cells in the synovial compartment express both receptors. In contrast to the anti-inflammatory type-2 T cells, type-1 cells express high levels of integrins and both CXCR3 and CCR5, which could contribute to the overrepresentation of this cell type in the synovial compartment. Whether DMARDs can affect the expression of any of those specific pro-inflammatory homing receptors is still not known.

The aim of this study is to investigate whether the various types of DMARDs can affect the phenotype and function of T cells in arthritis patients.

**Methods**

**Patients studied:**
Patients were recruited from the outpatient clinic for early arthritis of the Jan van Breemen Institute, Amsterdam. Diagnoses after one year of follow-up consisted of rheumatoid arthritis, undifferentiated arthritis and psoriatic arthritis. The diagnosis rheumatoid arthritis was made according to the ACR criteria. All patients diagnosed with undifferentiated arthritis had oligo- or polyarthritis. To study the in-vivo effects of DMARDs blood was obtained at the start of treatment and after 3 months of oral monotherapy with hydroxychloroquine (200mg/day), sulphasalazine (2000mg/day) or methotrexate (7.5 mg/week).

Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-hypopaque and stored in liquid nitrogen until use.

**Antibodies:**
Monoclonal antibodies (mab’s) directed against the following antigens were used in this study:

- *CLB, Amsterdam:* CD27-FITC (3A12), CD18, CD11a (Tb-133), CD2 (2H4, Hik-27), CD28 (15E8); *Becton Dickinson:* CD8-PerCP,IFN-γ-FITC, IL-4-PE; *Caltag:* CD4-APC; *Immunotech:* CCR5-PE; *R&D systems:* CXCR3

**T cell proliferation**
Whole blood of arthritis patients was diluted 1:10 with Iscove’s modified Dulbecco’s Medium Monoclonal antibodies directed against CD2 and CD28 were added for three days. Proliferation was measured by incorporation of tritium labeled thymidin and expressed as counts per minute (cpm).
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T cell phenotype
Four color flowcytometry was performed on 0.2 million thawed PBMC stained with directly labeled monoclonal antibodies (mab’s) against CD4 (APC) and CD8 (PerCP) simultaneously with directly labeled (FITC, Phycoerythrin) or indirectly (goat-anti-mouse phycoerythrin) mab’s directed against differentiation markers, adhesion molecules and chemokine receptors. Data were analyzed for CD4+ and CD8+ cells separately and expressed as percentages positive cells or mean fluorescence intensity (MFI).

Intracellular staining technique:
0.5 million PBMC were stimulated with 1 ng phorbol myristate acetate (PMA) (Sigma chemicals) and 1μM Ionomycin (Sigma chemicals) during 4 hours in the presence of 1 μM Monensin (Sigma chemicals). After incubation cells were stained with PerCP-anti CD8 and APC-anti CD4 mAb and subsequently fixed with 4% paraformaldehyde. Permeabilisation of the cells was done with 0.1% saponin for 45 minutes. Intracellular staining was performed with either IFN-γ-FITC and IL-4-PE or IL-2 FITC and IL-13-PE. Stained cells were analyzed in a FacsCalibur (Becton Dickinson) and expressed as percentages cytokine positive CD4+ and CD8+ cells.

Results

Patients:
43 arthritis patients were included, clinical characteristics are presented in table 1. Diagnoses did not change during the 3 months of follow-up. Patients using hydroxychloroquine or sulphasalazine had never used DMARDs before while methotrexate was used by patients not responding to sulphasalazine or hydroxychloroquine. Only patients were chosen that did not use DMARDs for 3 months during the start of methotrexate treatment.

Table 1. Clinical characteristics arthritis patients studied

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Hydroxychloroquine</th>
<th>Sulphasalazine</th>
<th>Methotrexate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean DAS* at start</td>
<td>5.3</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Mean DAS at 3 months of therapy</td>
<td>4.8</td>
<td>4.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Total number of patients</td>
<td>13</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>RA</td>
<td>8</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Psoriatic arthritis</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Undifferentiated arthritis</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
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</table>

* DAS =Disease activity score
**T cell proliferation**

Results are shown in Figure 1. Three months of treatment with hydroxychloroquine resulted in significant lower proliferative responses to anti-CD2 and anti-CD28 antibodies in contrast to treatment with sulfasalazine and methotrexate. These results show that although each DMARD is reported to diminish proliferative T cell responses in vitro, this can only be shown for hydroxychloroquine in vivo.

![Figure 1](image)

**Figure 1.**

Proliferative response in whole blood of arthritis patients after stimulation with anti-CD2 and anti-CD28 antibodies. Shown are counts per minute (CPM) before (closed bars) and after 3 months of DMARD treatment (open bars). Given are means and SEM, *: p<0.05

![Figure 2](image)

**Figure 2.**

Percentage of IFN-γ and IL-4 producing cells and their ratio within the CD4+ (A) and CD8+ (B) peripheral blood cells of arthritis patients, before (closed) and after 3 months of DMARD treatment (open). Given are means and SEM

*: p<0.05  **: p<0.01  ***: p<0.001
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Cytokine producing cells
All three DMARDs investigated have been shown to influence the type-1/type-2 T cell balance by decreasing the production of IFN-γ in vitro\textsuperscript{18,21,22}. To investigate whether this effect could be observed in vivo as well percentages of IFN-γ and IL-4 producing cells were determined in the peripheral blood of arthritis patients before and three months after DMARD treatment (figure 2). In patients treated with hydroxychloroquine or methotrexate no alterations were seen in percentages IFN-γ+ cells, IL-4+ cells or their ratio. However, a significant decrease in IFN-γ positive cells could be observed during sulfasalazine both in CD4+ and CD8+ T cells. The ratio between type-1 and type-2 cells was only significantly changed in CD8+ cells since within the CD4+ subset the percentage IL-4+ producing cells was decreased as well.

Expression of differentiation markers
Since changes in cytokine profiles during sulfasalazine could be a reflection of altered differentiation state of the T cells, flowcytometrical analysis for the expression of differentiation markers CD45RA and CD27 was performed. As shown in Table 2, subsets defined by these markers were not changed during treatment suggesting a direct cytokine suppressive effect of sulfasalazine.

Table 2. Percentages of different subsets of CD4+ and CD8+ T cells before and after 3 months treatment with sulfasalazine

<table>
<thead>
<tr>
<th></th>
<th>Start</th>
<th></th>
<th>3 months</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td></td>
<td>Start</td>
<td>3 months</td>
<td>Start</td>
<td>3 months</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>CD4+</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD45RA+CD27+</td>
<td>43.6</td>
<td>4.4</td>
<td>45.0</td>
<td>5.0</td>
</tr>
<tr>
<td>CD45RA-CD27+</td>
<td>42.6</td>
<td>3.5</td>
<td>42.2</td>
<td>3.7</td>
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<tr>
<td>CD45RA-CD27-</td>
<td>10.6</td>
<td>2.4</td>
<td>9.4</td>
<td>2.0</td>
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<tr>
<td>CD45RA+CD27-</td>
<td>3.3</td>
<td>0.8</td>
<td>3.3</td>
<td>0.9</td>
</tr>
<tr>
<td>CD8+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45RA+CD27+</td>
<td>41.7</td>
<td>4.8</td>
<td>42.1</td>
<td>4.9</td>
</tr>
<tr>
<td>CD45RA-CD27+</td>
<td>20.0</td>
<td>3.5</td>
<td>18.8</td>
<td>3.7</td>
</tr>
<tr>
<td>CD45RA-CD27-</td>
<td>5.2</td>
<td>0.8</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CD45RA+CD27-</td>
<td>33.1</td>
<td>6.1</td>
<td>34.1</td>
<td>24.1</td>
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</table>

* p = Wilcoxon signed rank test
Effect of DMARDs on the expression of adhesion molecules and chemokine receptors

The homing of leukocytes to the synovium is an essential step in the formation of the inflammatory infiltrate. This is mediated by the expression of integrins and chemokine receptors. Integrins are heterodimers of an α- and β chain and play a role in firm adhesion of the T cells to the synovial endothelium and extracellular matrix. Determination of the mean fluorescence intensity of two β chains during DMARD treatment revealed that only during sulfasalazine treatment the β2 chain (CD18, figure 3) was downregulated on both CD4+ and CD8+ cells while the expression of the β1 chain (CD29, figure 4) was not significantly changed by any of the DMARDs used. However, of the tested α chains associated with β1, CD49d was diminished on T cells of sulfasalazine treated patients (figure 4).

Methotrexate was the only DMARD that significantly influenced the expression of the chemokine receptor CCR5 on CD4+ and CD8+ cells, while CXCR-3 and CCR3 expression were not affected by any DMARD (figure 5).

Figure 3.

Mean fluorescence intensity (MFI) of CD18 on CD4+ (A) and CD8+ (B) peripheral blood cells of arthritis patients, before (closed bars) and after 3 months of DMARD treatment (open bars). Given are means and SEM. *: p<0.05
Figure 4.
Percentage of CD49 and CD29 positive cells within the CD4+ (A) and CD8+ (B) peripheral blood cells of arthritis patients, before (closed bars) and after 3 months of DMARD treatment (open bars). Given are means and SEM, *: p<0.05.
Figure 5.
Percentage of chemokine receptor positive cells within the CD4+ (A) and CD8+ (B) peripheral blood cells of arthritis patients, before (closed bars) and after 3 months of DMARD treatment (open bars). Given are means and SEM, *: p<0.05

Discussion:
In this study we investigated the effects of hydroxychloroquine, sulfasalazine and methotrexate on the phenotype and function of circulating T cells in arthritis patients. Although all three drugs are reported to decrease the proliferative responses of T cells in vitro\(^1\),\(^5\)-\(^7\),\(^29\),\(^30\), we only observed a significant inhibition of proliferation by hydroxychloroquine in vivo. Since the in vitro anti-proliferative effect of chloroquine is mediated by the inhibition of IL-2 production, we determined whether hydroxychloroquine treatment resulted in a decrease of IL-2 producing cells. However, we could not find a significant change in this cell type after 3 months of therapy.
Studies have shown that hydroxychloroquine can interfere with the process of antigen presentation\(^31\) and could therefore interfere in T cell differentiation. Flowcytometric analysis of circulating T cells before and during hydroxychloroquine treatment in our study however did not indicate a shift towards a less differentiated, more naive phenotype as
determined by CD45RA and CD27 expression. This finding suggests that the action of hydroxychloroquine in the treatment of arthritis is not mediated via reduced antigen presentation.

The interference of DMARDs with cytokine production of T cells has been widely suggested as a mode of action in arthritis. Despite the reported decrease in the production of the proinflammatory cytokine IFN-γ by T cells in the presence of different DMARDs in vitro, we show that only sulfasalazine was able to induce a significant reduction in IFN-γ producing T cells in arthritis patients. A possible explanation could be that sulfasalazine can interfere with IL-12 production by antigen presenting cells as was demonstrated in a mouse model. However we could demonstrate that in arthritis patients sulfasalazine reduced the IL-4 producing CD4+ cells as well making IL-12 not a likely target. Since IL-4 producers were not affected in the CD8+ subset it can be concluded that the balance between proinflammatory IFN-γ producing (type-1) and IL-4 producing (type-2) cells is shifted by sulfasalazine towards type-2.

Apart from the influence on proliferative and cytokine producing responses we investigated whether DMARD treatment could alter the expression of molecules on T lymphocytes involved in the homing to and retention in the synovium. Both β1 and β2 integrins are important in the adhesion to activated endothelium and the extracellular matrix in the synovium, while gradients of chemokines give direction to the T cells moving into the synovium. In this study we show that in sulfasalazine- in contrast to hydroxychloroquine and methotrexate treated patients, the expression of both the β2 integrins (CD18) and the β1 associated CD49d is diminished.

The finding of methotrexate being a moderator of CCR5 expression could point to a new mode of action for methotrexate in arthritis. CCR5 and CXCR-3 are the most abundantly expressed chemokine receptors in the rheumatoid synovium and they are also associated with interferon producing type-1 T cells. Therefore, although methotrexate does not influence the percentage of type-1 cells in the circulation of rheumatoid arthritis patients, it could influence the proportion of these cells in the synovial infiltrate.

In summary, in this study we show that although various overlapping effects of DMARDs on T cells are reported in vitro, distinct effects can be observed on the T cells in treated arthritis patients, which could account for synergistic anti-inflammatory action.

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