T cell differentiation in autoimmune diseases
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Chapter 3

Low percentages of IFN-\(\gamma\) producing T cells in Systemic Lupus Erythematosus

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Chapter 3

Abstract

It has been suggested that the balance between IFN-γ (type-1) and IL-4 (type-2) producing T cells plays a role in the induction and perpetuation of autoimmunity. This balance was reported to be altered in the peripheral blood of SLE patients due to a low percentage of IFN-γ producing T cells. The purpose of this study was to quantitate this balance and investigate whether alterations in the type1/type-2 ratio are related to changes in the composition of the circulating T-cell compartment.

Methods: Peripheral blood was obtained from SLE patients and healthy matched controls. IFN-γ and IL-4 producing T cells (CD3+) were enumerated after short stimulation using an intracellular staining technique and flow cytometric analysis. Next to this the differentiation state of the peripheral blood T cells was evaluated.

Results: Both CD8- and CD8+ T cells of SLE patients contained lower percentages of IFN-γ producing cells than age- and sex matched healthy controls. Since percentages of IL-4 positive CD8- cells did not differ between both groups the IFN-γ/IL-4 (Th1/Th2) ratio was significantly lower in SLE patients. After in culture of PBMC with anti-CD2 and anti-CD28 antibodies, IFN-γ producers stayed significantly lower in patients, but after addition of exogenous IL-12 (1 ng/ml) the difference disappeared. Phenotypic analysis of freshly isolated T cells revealed that low percentages of IFN-γ producing T cells in SLE patients could not be ascribed to differences in memory typed CD45RA-CD95+ cells between the two groups. Interestingly, significantly lower percentages of terminally differentiated CD4+CD27- cells were found in SLE patients.

Conclusion: Low percentages of IFN-γ producing CD8- and CD8+ T cells were found in the peripheral blood of SLE patients in comparison with healthy controls. Circulating SLE T cells showed signs of diminished differentiation but could still be directed into IFN-γ producing cells when stimulated in an IL-12 rich environment.

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic remitting-relapsing disease, which can affect virtually any organ. The origin is believed to be multifactorial resulting in systemic B-cell hyperactivity and production of pathogenic autoantibodies. T cells appear to play a role since specific clones can be isolated from patients that can drive B cells to produce pathogenic anti-DNA autoantibodies. Furthermore intrinsic T cell abnormalities have been described in SLE patients. Special attention has been paid to the balance between the interferon-γ (IFN-γ) producing type-1 and interleukin-4 (IL-4) producing type-2 T cells in SLE. An imbalance between these two subsets is believed to play a role in the development and progression of autoimmunity in general. Several reports suggest that in SLE there is an imbalance in favour of the type-2 cells since there is an impaired IFN-γ production by peripheral blood T lymphocytes. The differentiation of T cells into either the type-1 or type-2 direction depends on many factors but the production of cytokines by antigen presenting cells (APC)
IFN-γ producing T cells in SLE

appears to play a role. APC derived IL-12 is a potent inducer of IFN-γ production while IL-10 gives rise to type-2 T cells partly by antagonizing effects on IL-12 production. Several reports have shown that in SLE there are increased IL-10 levels\textsuperscript{12-14} which could explain the suppression of IFN-γ production.

Despite the low production by peripheral blood cells, IFN-γ has been shown to be a contributing factor in the development of tissue damage in murine models\textsuperscript{15-17} while IFN-γ treatment can exacerbate human SLE\textsuperscript{18,19}. This paradox could be explained by the assumption that peripheral blood T cells of SLE patients can still differentiate into pathogenic type-1 cells after entering the tissues. Therefore we investigated the differentiation state of peripheral blood T cells of SLE patients in relation to the type-1/type-2 balance. We found that decreased percentages of IFN-γ producing CD4+ T cells in the peripheral blood of SLE patients may be related to an underrepresentation of terminally differentiated CD4+ cells lacking the CD27 antigen\textsuperscript{20} in the blood. Moreover, both CD4+ and CD8+ SLE T cells were capable of differentiating into adequate IFN-γ producing cells in the presence of IL-12 in vitro.

Materials and methods

Study population. Of 8 SLE patients and 8 healthy controls peripheral blood (PB) was obtained in heparinized tubes and diluted in phosphate buffered saline (PBS) containing 10% fetal calf serum (FCS) and 5% trisodiumcitrate. 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.

Intracellular staining technique. Cells positive for IFN-γ and IL-4 were determined by flowcytometry. In short, mononuclear cells were stimulated with 1 ng phorbol myristate acetate (PMA) (Sigma chemicals) and 1μM Ionomycin (Sigma chemicals). 1 μM Monensin (Sigma chemicals) was added to prevent excretion of the newly synthesized cytokines. After 4 hours of incubation cells were stained with PE-anti CD3 and FITC-anti CD8 monoclonal antibodies (mAb) (both Becton and Dickinson, Mountain View, CA). Cells were fixed with 4% paraformaldehyde. Cells were stored overnight in PBS at 4°C. Permeabilisation of the cells was done with 0.1% saponin supplemented with 10% pooled human serum (HPS) to block aspecific binding of mAb. Directly phycoerythrin (PE) labeled anti-IFN-γ, anti-IL-4 and a negative control (all Becton Dickinson) were diluted in PBS/BSA/Saponin to a concentration of 5μg/ml and added to the cells in aliquots of 50ml for 25 minutes at 4°C. Cells were analyzed on a FACScan (Becton Dickinson). IFN-γ and IL-4 containing CD8- (defined as CD4+) and CD8+ T cells were identified by gating for CD3 positivity and setting a marker using the sample containing the negative control antibody. Cytokine producers were expressed as percentages of CD3+CD4+ and CD3+CD8+ T cells.
Induction of IFN-γ producing T cells by IL-12. 0.4 million PBMC were stimulated with 1:1000 ascites containing anti-CD2 antibodies (CLB T11.1/1, CLB T11.2/1, Hik-27, CLB, Amsterdam, The Netherlands) and anti CD28 (CLB-CD28/1, CLB, Amsterdam, The Netherlands) in the presence or absence of recombinant IL-12 (1 ng/ml) for four days. Then cells were restimulated with PMA and ionomycin for 4 hours in the presence of Monensin and stained for intracellular IFN-γ as described above.

Determination of the differentiation antigens on peripheral blood T cells. Three-color flowcytometric analysis was performed on 0.2 million freshly isolated mononuclear cells with combinations of anti CD95-FITC (Immunotech), anti CD45RA (2H4, Coulter), anti CD27-FITC (3A12, CLB, Amsterdam, The Netherlands), anti CD4-PerCP and anti CD8-PerCP (both Becton Dickinson) in 100 µl PBS/0.05% BSA. Cells were incubated for 20 minutes at 4°C.

Production of monokines. Whole blood of patients and controls was diluted in LPS free IMDM (1:10). 100 pg/ml lipooligosacharide (LOS) was added with thromboliquine. After incubation for 24 hours cells and supernatant were stored at -20°C. IL-12 (p40), IL-10 and IL-6 were determined in supernatant with ELISA (CLB, Amsterdam, The Netherlands).

Results

Clinical features

Eight patients with SLE with low disease activity and eight healthy controls were included in this study. All patients fulfilled the 1982 ARA criteria for SLE. Demographic data of the patients included in the study are presented in Table 1. Three patients were using prednisone (3, 4 and 30 mg/day). In addition one of them used 200mg azathioprine daily.

Table 1. Characteristics of population studied

<table>
<thead>
<tr>
<th></th>
<th>SLE</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/Male</td>
<td>7/1</td>
<td>7/1</td>
</tr>
<tr>
<td>Age* (years)</td>
<td>37.9 ± 12.7</td>
<td>41.1 ± 10.5</td>
</tr>
<tr>
<td>Disease duration* (years)</td>
<td>7.9 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>Disease activity* (SLEDAI)</td>
<td>3.8 ± 2.9</td>
<td></td>
</tr>
</tbody>
</table>

*= Mean ± standard deviation

Quantitation of IFN-γ and IL-4 producing T cells

To determine whether the percentages of type-1 and type-2 cells in the peripheral blood of SLE patients were different from healthy controls, intracellular cytokine staining experiments were performed and presented in Figure 1. In agreement with previous reports,
lower percentages of IFN-γ producing CD3+CD8- T cells (Th1) were found in SLE patients in comparison with healthy controls. However IL-4 producing CD3+CD8- (Th2) cells were not significantly different between the two groups. The Th1/Th2 ratios were therefore lower in SLE patients. Within the CD8+ T cell compartment lower percentages of IFN-γ producing cells were found as well. IL-4 positive CD8+ T cells were only detectable in 2 SLE patients and 3 healthy controls.

**Phenotypic analysis of T cells in patients and controls**

To determine whether the decreased percentage of IFN-γ producing cells could be explained by the differentiation state of T cells in SLE patients, phenotyping for the differentiation antigens CD45RA, CD95 and CD27 was performed with simultaneous analysis of CD4 and CD8 expression. After gating for CD4+ and CD8+ cells, percentages of “naive” CD45RA+ CD95-, CD45RA+CD95+ and “memory” CD45RA-CD95+ cells were not different between SLE patient and healthy controls. However significant lower percentages of terminally differentiated CD4+CD27- cells were found in SLE patients than
in healthy controls (table 2). The percentage of CD27- cells however was not different for CD8+ cells.

_Differentiation of Type-1 cells in vitro_
To investigate whether peripheral blood T cells of SLE patients were able in vitro to enhance IFN-γ (type 1) production after prolonged activation, mononuclear cells of patients and controls were cultured in vitro in the presence and absence of recombinant IL-12 (table 3). After four days of culture still lower percentages of IFN-γ producing cells were found in SLE patients in both CD8- and CD8+ subsets. However in the presence of exogenous IL-12 no significant difference between SLE patients and controls could be found any more after 4 days.

_Production of monokines in whole blood_
Since the capacity to differentiate in type-1 cells of SLE peripheral blood T cells seems to be normal in the presence of IL-12, low IFN-γ production could be caused by a diminished production of IL-12 or an overproduction of IL-10 which is antagonizing IL-12. Therefore we investigated whether the capacity of T cells to produce IFN-γ was related to the capacity to produce IL-12 or IL-10 in these SLE patients. Diluted whole blood was stimulated with LOS and production of the cytokines IL-12 (p40), IL-10 and IL-6 was determined in the supernatant after 24 hours. As presented in table 4, no significant differences were found for either of these cytokines between SLE patients and controls nor did they correlate with IFN-γ production.

| Table 2. Percentages of cells positive for differentiation markers in the peripheral blood of SLE patients and matched healthy controls |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                | **CD4+**                        | **CD8+**                        |                                |
|                                | Median  | Range  | Median  | Range  |
| SLE CD45RA+CD95-               | 50.3    | 24.9 - 75.7 | 43.8    | 20.6 - 74.6 |
| Controls                       | 40.3    | 5.1 - 70.3 | 52.2    | 10.1 - 83.4 |
| SLE CD45RA+CD95+               | 14.0    | 6.7 - 39.5 | 45.7    | 21.7 - 59.5 |
| Controls                       | 17.1    | 9.7 - 22.4 | 32.5    | 13.6 - 56.8 |
| SLE CD45RA-CD95+               | 23.6    | 16.9 - 42.4 | 11.4    | 1.6 - 19.7 |
| Controls                       | 40.3    | 17.7 - 75.0 | 14.3    | 2.6 - 32.5 |
| SLE CD27-                      | 2.1     | 1.6 - 10.0 | 8.3     | 1.3 - 31.3 |
| Controls                       | 6.7*    | 1.8 - 14.0 | 9.1     | 2.7 - 22.2 |

* p<0.05 by Mann-Whitney test for SLE vs. controls
IFN-γ producing T cells in SLE

Table 3. Percentage of IFN-γ positive cells in cultured PBMC stimulated with anti-CD3 and anti-CD28 antibodies in the absence or presence of IL-12 for 4 days

<table>
<thead>
<tr>
<th>CD4+</th>
<th>CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median range</td>
<td>Median range</td>
</tr>
<tr>
<td><strong>Medium</strong></td>
<td><strong>IL-12</strong></td>
</tr>
<tr>
<td>SLE</td>
<td>12.3</td>
</tr>
<tr>
<td>Controls</td>
<td>19.8*</td>
</tr>
<tr>
<td>SLE</td>
<td>43.5</td>
</tr>
<tr>
<td>Controls</td>
<td>55.6</td>
</tr>
</tbody>
</table>

*p<0.05 with the Mann-Whitney test for SLE vs. Controls

Table 4. Production of monokines by LOS stimulated wholeblood of SLE patients and healthy matched controls

<table>
<thead>
<tr>
<th>Monokine</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE IL-10</td>
<td>141</td>
<td>4 - 403</td>
</tr>
<tr>
<td>Controls</td>
<td>81</td>
<td>1 - 221</td>
</tr>
<tr>
<td>SLE IL-12 (p40)</td>
<td>414</td>
<td>100 - 2490</td>
</tr>
<tr>
<td>Controls</td>
<td>574</td>
<td>12 - 952</td>
</tr>
<tr>
<td>SLE IL-6</td>
<td>1711</td>
<td>962 - 5407</td>
</tr>
<tr>
<td>Controls</td>
<td>1548</td>
<td>1 - 5168</td>
</tr>
</tbody>
</table>

Discussion

In the pathogenesis of SLE a dysbalance in the type-1/type-2 T cells is suggested. In the peripheral blood of SLE patients decreased IFN-γ production has been documented in previous reports suggesting a Th2 dominance in the pathogenic process. We here confirm these results in patients with low disease activity, both in the CD8- and CD8+ T cell compartments. Although three SLE patients were taking immunosuppressive drugs these were not responsible for this finding. It has been suggested that the lower percentage of IFN-γ producing T cells in the circulation of SLE could result from low production of
IL-12\textsuperscript{10,21-23} or by overproduction of by IL-10\textsuperscript{8,13,14,23-25} during active disease. IL-10 is a well-known downregulator of IL-12 p40 production\textsuperscript{26,27}. In our patient group, with low disease activity, both IL-10 and IL-12 p40 production as well as their ratio were not different in comparison with healthy controls. In addition no significant correlation was observed between IL-12 p40, IL-10 production or their ratio and Th1/Th2 ratio measured. This suggests that the lower percentage of IFN-γ producing T cells in these patients is not caused by an ongoing altered production of IL-12 or IL-10. However, it could that during periods of disease activity high production of IL-10 can interfere with the development of type-1 T cells.

Both IFN-γ and IL-4 are produced by differentiated “memory” (CD45RA-CD45RO+CD95+) T cells. SLE patients are reported to have a higher proportion of memory type T cells than healthy controls especially during active disease\textsuperscript{28}. Therefore the observation of low percentage of IFN-γ could be due to low percentages of memory T cells. Phenotypic analysis however showed that SLE patients in this study had no significant different proportion of CD45RA- T cells in both CD8- and CD8+ compartments than healthy controls, probably due to low disease activity. Neither were any differences found in CD95+ cells between patients. This was a surprising observation since CD4+CD45RA+ cells in Japanese SLE patients are reported to be CD95 positive\textsuperscript{29}. This discrepancy could be caused by a selection of different patients considering that we found high percentages of CD45RA+CD95+ cells in a patient with primary Sjögren syndrome (data not shown).

Despite normal percentages of memory typed cells, significant lower percentages of CD4+CD27- cells were found in SLE patients. CD4+CD27- cells are highly differentiated since they are generated after prolonged stimulation of CD4+CD45RO+CD27+ cells in vitro\textsuperscript{20,30}. Within the CD4+CD27- subset more IFN-γ and IL-4 producers are found than in the CD4+CD27+ subset\textsuperscript{31}. Lower percentages of CD4+CD27- cells in this patient group could therefore suggest that CD4+ cells in inactive SLE patients are less differentiated than that of healthy controls and could explain the low percentages of IFN-γ producing cells. Both with respect to homing receptor and chemokine receptor expression CD4+CD27- T cells are distinct from recirculating CD4+CD45RO+CD27+ memory T cells\textsuperscript{32}. Therefore the low abundance of CD4+CD27- T cells in the circulation of SLE patients could be comparable with an increased representation of these cells in affected tissue.

Although there is a Th1 deficiency in the peripheral blood of SLE patients in murine SLE studies it has been shown that there is a local production of IL-12 in the kidney\textsuperscript{33} and that IFN-γ can accelerate the development of clinical symptoms\textsuperscript{34}. This suggests that the local induction of type-1 cells can be involved in the development of the process of tissue damage in SLE. Since the IFN-γ deficient T cells in the circulation of SLE patients are not differentiated into type-2 effector cells as shown here by normal percentages of IL-4 producing cells, the capacity to produce IFN-γ after they leave the circulation could be important for a local Th1 dominated reaction. In this study we could demonstrate that after prolonged stimulation in vitro, the increase in IFN-γ producing cells is still lower in SLE patients than in healthy controls. However activation in the presence of IL-12 achieves percentages of IFN-γ producing CD8+ and CD8- T cells that are not significantly different between SLE patients and healthy controls.
Conclusion

Low IFN-γ production by mononuclear cells of SLE patients in vitro is often explained by the hypothesis that T cells are exhaustedly stimulated in vivo. Our results suggest that peripheral blood T cells in SLE despite low production of IFN-γ can be induced to yield higher percentages of IFN-γ when activated in an IL-12 rich environment. Therapeutic use of IL-12 might therefore result in an increase in IFN-gamma producing cells in the peripheral blood. However it could on the other hand amplify a localized type-1 response leading to increased tissue damage in affected organs.

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