CD27+ memory and CD27- effector CD8+ T cells are responsible for a decreased production of proinflammatory cytokines in HLA B27-positive subjects

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Abstract

Background and objective

AS and other spondyloarthritides (SpA) are mostly chronic inflammatory rheumatic diseases characterised by a strong association with HLA B27. Recent data from our group have suggested that AS patients have a diminished secretion capacity of inflammatory cytokines, possibly associated with HLA B27. The aim of this study was to identify CD4+ and CD8+ T cell subsets responsible for the observed lower cytokine secretion capacity in HLA B27-positives.

Methods

Highly purified (> 98%) CD4+ and CD8+ T cells of HLA B27-positive AS patients (n = 13), healthy HLA B27-positive (n = 7) and -negative controls (n = 9) were stimulated for 6h with PMA/Ionomycin and, after fixation, stained for surface markers CD45RA and CD27 and cytokines TNFα, IFNγ, IL-4 and IL-10.

Results

CD27+ CD45RA- memory CD8+ T cells of HLA B27-positive subjects showed a significantly lower percentage of TNFα (median 71.4%) and IFNγ production (median 69.7%) than HLA B27-negative controls (TNFα 85.1%; p ≤ 0.027; IFNγ 82.7%; p ≤ 0.026). A similar result was also detected in CD27- CD45RA+ effector CD8+ T cells of which 43.2% produced TNFα and 66.3% IFNγ in HLA B27-positive subjects, respectively, compared to 75.6% TNFα and 84.4% IFNγ producing T cells in HLA B27-negatives (p ≤ 0.045 and p ≤ 0.062, respectively). For all CD4+ T cell subsets no significant differences between HLA B27-positive and HLA B27-negative donors were observed, regarding neither the frequency of IFNγ, TNFα, IL-4 or IL-10 producers nor the coexpression of IFNγ and IL-4 in memory subsets.

Conclusions

HLA B27-positive subjects are characterized by a low proinflammatory cytokine production in CD8+ effector and memory T cell subsets. This suggests an influence of HLA B27 on cytokine production in antigen-experienced CD8+ T cells.

Key words

TNF-alpha, IFN-gamma, HLA B27, ankylosing spondylitis, T cells.
T-cell subsets in HLA B27-positives / S. Kohler et al.

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Introduction

Ankylosing spondylitis (AS) is one of the most frequent chronic inflammatory rheumatic diseases (1). About 90% of Caucasian AS patients are HLA B27 positive (2) and 20-50% of the genetic risk to develop AS is HLA B27 associated (3). The finding that AS is a polygenic disease has lead to a more intensive examination of genes located close to the B27 gene on chromosome 6, of special interest is the about 250kb distance from B27 located TNFα promoter region (4). Independent of an MHC class I or II association, there are a number of TNF polymorphisms in this region which seem to contribute differently to the susceptibility and severity of infectious diseases such as cerebral malaria (5), leishmaniosis (6), meningococcal disease (7), and autoimmune diseases such as multiple sclerosis (8) and rheumatoid arthritis (9). There are some indications that the allelic variants of the TNF promoter at positions -238 and -308 are associated with a different production of TNFα by T cells and macrophages (10, 11). Comparisons of the frequencies of these allelic variants in HLA B27-positive AS patients and in HLA B27-positive and negative healthy donors brought conflicting results (12-15). Using advanced flow cytometric technology we found a decreased TNFα secretion capacity in patients with chronic reactive arthritis (16). This was similar in AS, where a decreased T cell TNFα and IFNγ production of HLA B27-positive AS patients and healthy controls compared to HLA B27-negative healthy donors was detected, this was partly associated with TNF genotypes (17). Thus, HLA B27-positive patients with AS and other spondyloarthritides seem to have a lower capacity to secrete TNFα and IFNγ than HLA B27-negatives.

The aim of the present study was to further characterize these T cells by definition of subpopulations and their cytokine production. Such a systematic analysis of the cytokine secretion capacity of CD4+ and CD8+ T cells becomes possible by using a combination of magnetic cell separation and 4-colour-flow cytometry. This allows staining of intracellular cytokines and in parallel surface markers such as CD27 and CD45RA, which define phenotypically and functionally distinct T cell subsets.

CD4+ T cells lacking CD27 expression represent specialized CD4+ memory T cells, which arise during persistent antigenic stimulation (18). These differentiated memory CD4+ CD27+ cells, which almost exclusively reside within the CD45RA- CD45RO+ subset, have an enhanced capacity to secrete cytokines (19-23). In accordance, proliferative responses to tetanus toxoid and allergens in atopic individuals were mainly confined to this population (18). Additionally, the multiple stimulation of CD27-memory CD4+ T-cells may also be accompanied by an enhanced differentiation into TH1 or TH2 cells. While this might lead to a negative association of IFNγ and IL-4 production, it has not been shown so far.

Naive CD8+ T cells resemble naive CD4+ T cells not only with respect to their cytokine production but also regarding their expression of surface markers CD45RA and CD27. On the contrary CD8+ CD45RA+ CD27 T cells, which are more frequent than their CD4+ counterparts, are characterized by a specific expression pattern of surface markers, the production of effector cytokines and strong cytolytic activity. These cells are regarded as effector cytotoxic T cells, which induce apoptosis of target cells by exocytosis of granula as well as by Fas/FasL interaction (24, 25). As opposed to CD45RA- CD27+ cells, CD45RA- CD27+ CD8+ T cells, which express less granzyme B and perforin and display a weak cytolytic activity, are regarded as CD8+ memory cells (24). The subpopulation of CD45RA- CD27- CD8+ T cells is usually small and supposed to contain both memory and effector-type cells.

Interestingly, several recent studies have provided data that point to a significant role of CD8+ T cells in the pathogenesis of AS and other spondyloarthritides. Thus, CD8+ T cells in the synovial fluid express high levels of the activation marker HLA-DR (26), and the percentage of CD8+ CD28- T cells is higher in AS patients compared to controls (27). On the background that there are gener-
ally more differences in the Vβ repertoire of CD8+ than of CD4+ T cells (28), an increased oligoclonality of the CD8+ T cells has been reported in AS twin pairs (29).

On this basis, we analyzed the T cell subpopulations of HLA B27-positive AS patients and HLA B27-positive and HLA B27-negative healthy donors and assessed their cytokine secretion patterns to further clarify the question of the decreased TNFα secretion repeatedly found in HLA B27-positives.

Material and methods

Patients
The patients in this study were attending the rheumatology outpatient clinic of the Campus Benjamin Franklin, Charité Berlin, Germany. All AS patients had an active disease with a disease activity index (BASDAI) > 3.0 and fulfilled the 1984 criteria for the disease (30). To avoid possible interferences, patients receiving immunosuppressive drugs or corticosteroids were not included in the study. Informed consent was obtained from all blood donors and the study was approved by the local ethical committee.

Table I shows the characteristics of the 13 patients with AS (all HLA B27-positive), 7 HLA B27-negative and 9 HLA B27-negative healthy controls. Patient and control samples were randomly collected, processed and stained.

T cell purification

PBMCs were separated by the centrifugation of 50ml heparinized peripheral blood on Ficoll-paque (Pharmacia, Uppsala, Sweden) and subsequent washing with PBS/BSA 0.5%. The separation of CD4+ and CD8+ T cells was performed with MidiMacs columns (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s instructions. In brief, PBMCs were divided in 2 equal portions, which were then incubated with magnetic CD4 and CD8 Multisorbsbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), respectively. After the removal of unbound beads by washing, the cells were pipeted on magnetic columns. In this process the positive fraction is retained in the magnetic field and can later be harvested. After separation, surface staining for CD4 and CD8 was performed in case of a significant (>20%) fraction of CD4+CD8+ double-positive cells, they were depleted in an additional step. Final purity exceeded at least 98% in all samples.

T cell stimulation and staining

The cells were stimulated with PMA (Phorbol 12-myristate 13-acetate; Sigma, St Louis, MO; 5ng/ml) and ionomycin (Sigma, St Louis, MO; 1μg/ml) for 6h in 1ml wells at a concentration of 1x10^6/ml in RPMI medium supplemented with 10% FCS (Fetal cell serum, PAA, Linz, Austria) and penicillin (100U/ml) and streptomycin (0.3mg/ml). Brefeldin A (5μg/ml) was added for the last 2h and, after 2 washing cycles with PBS, the cells were fixed in 2% formaldehyde and stained. Importantly this procedure does not affect the surface expression of CD27 and CD45RA.

Surface staining was performed by the incubation of cells with phycoerythrin (PE) or Cy5 labelled anti-CD27 [clone 2E4, a generous gift from R. van Lier (Amsterdam, Netherlands)] and Biotin coupled anti-CD45RA (Becton Dickinson, Heidelberg, Germany) for 10 minutes. After washing cells were incubated with PerCP coupled streptavidine, washed and permeabilized with 0.5% saponin (Sigma, St Louis, MO), followed by intracellular staining of cytokines performed in pairs containing either anti-IFNγ and anti-IL-4 or anti-TNFα and anti-IL-10 mAbs, respectively. The following mAbs were used: Cy5 or FITC coupled anti-IFNγ (4SB3), PE coupled anti-IL-4 (4D9), Holzel Diagnostica, Cologne, Germany), PE or Cy5 coupled anti-IL-10 (Pharmingen, San Diego, California, USA) and FITC coupled anti-TNFα (Hölzel Diagnostica, Cologne, Germany). FACS analysis was performed using CellQuest software (Becton Dickinson, Palo Alto, California, USA), see example Fig. 1. According to their expression of CD45RA and CD27, CD4+ T-cells were grouped into CD45RA/CD27- “naive CD4+”, CD45RA/CD27- “memory CD4+” and CD45RA/CD27+ “differentiated memory CD4+” T-cells. CD45RA/CD27- CD8+ T-cells will be referred to as “naive CD8+”, CD45RA/CD27- as “memory CD8+” and CD45RA/CD27+ as “effector CD8+” T-cells.

Statistical analysis

The observed value for IFNγ and IL-4 coexpressing cells in percentage was compared to the expected value calculated for random coincidence of two independent variables. The correlation of cytokine coexpression in total was calculated using the test for ψ-correlation coefficients (31). The comparison of ψ-correlation coefficients and cytokine production in T cell subsets was performed by the two-tailed Wilcoxon test. For the analysis of statistical differences in cytokine production between HLA B27-positive and HLA B27-negative donors the Mann Whitney U-test was used. The Mann Whitney U-test was used since the number of samples examined did not allow the assumption of a normal distribution. In this test the use of medians rather than means compensates for extreme values.

All tests were calculated using SPSS software. P-values ≤ 0.05 were considered as statistically significant, p-values ≤ 0.075 were regarded as a trend for statistical significance.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (y)</th>
<th>M/F ratio</th>
<th>Disease duration (y)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA B27-negative AS patients</td>
<td>38y</td>
<td>5/4</td>
<td>7.8y</td>
<td>Diclofenac; sulfasalazine</td>
</tr>
<tr>
<td>HLA B27-positive AS patients</td>
<td>46y</td>
<td>1/6</td>
<td>7.8y</td>
<td></td>
</tr>
</tbody>
</table>

(mean: |
Results

Differences in cytokine production between HLA-B27-positive and -negative donors

It was possible to perform a detailed analysis of cytokine expression in different T cell subsets because CD4+ and CD8+ T cells were separated with at least 98% purity before stimulation with PMA/Ionomycin, which was followed by surface staining for CD27 and CD45RA and intracellular cytokines. Since, in concordance with previous work (17), no differences in cytokine production between HLA B27-positive healthy donors and HLA B27-positive AS patients were noticed, it was decided to compare the combined group consisting of AS patients and healthy donors with HLA B27-negative healthy individuals. In contrast to (17) in all CD4+ T cell subpopulations no significant differences in TNFα, IFNγ, IL-4 or IL-10 production after polyclonal T cell stimulation could be detected between HLA B27-positive and HLA B27-negative donors (data not shown). However, in CD8+ T cells the memory as well as the effector compartment showed marked differences in proinflammatory cytokine production: in HLA B27-positive individuals a significantly lower percentage of memory CD8+ T cells produced TNFα (median 71.4% vs. 85.1%; \( p \leq 0.027 \)) and IFNγ (69.7% vs. 82.7%; \( p \leq 0.026 \)) compared to HLA B27-negative healthy controls (Fig. 2). For TNFα this was also observed in effector CD8+ T cells (43.2% vs. 75.6%; \( p \leq 0.045 \)), with a trend for a reduced IFNγ-production (66.3% vs. 84.4% \( p \leq 0.062 \)) in HLA B27-positive compared to HLA B27-negative donors (Fig. 3). Interestingly, amongst antigen inexperienced, naive CD8+ T cells of HLA B27-positive and -negative donors, no significant differences regarding the frequency of TNFα and IFNγ producers could be detected (TNFα 7.4% vs. 6.3% \( p \leq 0.6 \), IFNγ 6.5% vs. 3.1% \( p \leq 0.1 \)). The production of cytokines IL-4 and IL-10 by CD8+ T cells was very low, as expected, and did not show any differences (data not shown).

Subpopulation distribution in patients suffering from AS compared to healthy persons

A comparison of HLA-B27 positive AS patients, healthy HLA-B27 negative and positive individuals showed no significant differences in the distribution of CD4+ and CD8+ T cell subsets defined by CD27 and CD45RA surface expression in the three donor groups. Additionally, we could not detect significant differences in the frequencies of these subsets in male or female donors (data not shown).

TH1/TH2 coexpression pattern of different CD4+ T cell subsets

The observed lower cytokine production in memory and effector CD8+ T cells might be associated with a differ-
entiated memory CD4+ T cells there was a significant reduction in the percentage of IFN-γ secretion capacity in HLA B27-positive donors (median $\phi = -0.15$, range -0.06 to -0.46) as compared to HLA B27-negative individuals, and this could also be observed as a clear trend in CD8+ effector T cells. On the basis of this data, we hypothesize that the differentiation pathway from naive to memory/effector CD8+ T cells may be altered in HLA B27-positive individuals.

The main aim of our study was to identify the peripheral blood T cell subsets, which are responsible for the low TNF-α and IFN-γ secretion capacity previously reported (17). In concordance with that, HLA B27-positive healthy donors and AS patients did not differ with regard to their subset specific cytokine secretion capacity in the present study. However, in contrast to the previous study, no generally compromised capacity to secrete TNF-α was found in HLA B27-positives. The deficiency was exclusively seen in CD8+ memory and effector T cells, while no significant differences were found in CD4+ and naive CD8+ T cells. Furthermore, in contrast to the previous study (17), in this study a generally higher frequency of cytokine producers was detected in CD4+ and CD8+ T cells. These differences can be largely explained by the different methodology used: the stimulation of freshly isolated rather than frozen cells may well account for the higher cytokine production we observed. In addition, the method of separation and stimulation of highly purified CD4+ T cells in this study allows for a more accurate estimation of CD4+ cytokine secretion, excluding direct or indirect influences of other cell types. Moreover, the indirect identification of CD4+ T cells after PMA/Ionomycin stimulation by CD3 and CD8 staining is unnecessary. Therefore, the possibly confounding effect of CD3+ CD4+ CD8- and CD3- CD4+CD8+ cells, which were depleted during the sorting procedure, can be excluded.

This study suggests that HLA B27 is involved in the diminished cytokine production of distinct CD8+ T cell subsets. However, the reason for this is not clear at present. Apparently, as only memory and effector CD8+ T cells, but not naive CD8+ and no CD4+ T cell subsets were compromised, the low TNF-α production does not appear to be...
intrinsic, but rather a defect acquired during T cell differentiation from naive into memory and effector CD8+ T cells in HLA B27-positive individuals. The fact that not only the frequency of TNFα, but also of IFNγ expressing CD8+ T cells was reduced, together with the obvious importance of interactions of CD8 with MHC class I in CD8+ T cell differentiation, supports this assumption. However, direct evidence for an involvement of HLA B27 in CD8+ T cell differentiation and for the mode of action remains to be shown.

In the previous study (17) and in this one, no difference between HLA B27-positive healthy donors and AS patients was found. Since 95% of the AS patients are HLA B27 positive and most HLA B27 subtypes are associated with the disease (32), this surface molecule seems to be rather critical for the development of AS. However, from family studies it is known that only less than 30% of the total genetic load of AS is due to HLA B27 (3), and it also seems clear that at least 80% of the normal HLA B27-positive population remain healthy (1). This is consistent with the hypothesis of an HLA B27-related lower cytokine secretion that is strongly involved, but on its own is not sufficient for the development of the disease. Another feature that may play a role in SpA are infections as triggering or perpetuating factors. On the one hand, there is no evidence so far, that HLA B27-positive individuals have any clinically relevant immune deficiency associated with an increased risk of infections (33). This would indicate that the relatively diminished cytokine production of HLA B27-positive individuals observed may not be functional. On the other hand, there are other clear indications that HLA B27 is associated with infections leading to arthritis as in reactive arthritis (ReA), which occurs after infections with pathogens such as chlamydia, yersinia and salmonella (34), and in the HLA B27 transgenic rat model where germs are needed to induce an SpA-like disease occurring in these animals (35-37). Non HLA B27-associated SpA is common among HIV infected patients in Zambia (38), which suggests a link between compromised immune competence and SpA. In ReA patients, we found a low TNFα secretion in patients with more severe disease and longer disease duration (16).

In general, a lower capacity of cytokine secretion might lead to a handicap in host defence against intracellular pathogens, which is predominantly generated by CD8+ T cells. Although this handicap does not seem to strongly affect the final clearance of the pathogen (33), this may lead to a longer persistence of infectious agents and facilitate molecular mimicry or other autoimmun mechanisms considered to be implicated in the onset of the disease. The difference between an HLA B27-positive healthy subject and an HLA B27-positive AS patient may not be a lower capacity to secrete proinflammatory cytokines but rather the encounter of certain infectious agents under circumstances as yet not defined.

In contrast to the lower cytokine production we have seen in HLA B27 positive individuals, TNFα has been detected in the synovial joint of AS patients (39) and clear positive effects of anti-TNFα antibodies on AS disease activity have been reported (40). However, there might be a discrepancy between the cytokine secretion capacity of PB T cells, sytovial T cells and, furthermore, macrophages which might be the most abundant TNFα secreting cells at the site of inflammation. This study shows that the TNFα expression of CD4+ and CD8+ T cells is not regulated equally. Thus, TNFα secretion of T cells may well have a regulatory function in the immune response, which may play a role in the onset of AS in connection with an as yet undefined infectious process. But how the magnitude of TNFα secretion of T cells affects local inflammation in established disease remains to be clarified.

Reports about clonal expansions of CD8+ and CD4+ T cells in AS peripheral blood (29) and psoriatic arthritis joint fluid (41) might indicate that a chronic inflammatory disease like AS is characterized by an expansion of disease mediating T cell subsets. Therefore we compared the percentages of T cell subsets distinguished by CD45RA and CD27 expression in the peripheral blood of healthy subjects and patients. Since we could not find any differences in the three groups, our study does not add any evidence in favour of an imbalance in the memory subsets of AS patients. With respect to the oligoclonality reported for CD8+ T cells in AS, it is unclear whether this leads to changes in the subset markers examined in this study. Furthermore, it cannot be excluded that AS disease sustaining T cells are trapped in the inflamed tissue and do not recirculate in sufficient numbers to effectively change the subset distribution in PB (29, 42).

Though the comparison between HLA B27-positive and HLA B27-negative individuals revealed no significant differences regarding the frequency of cytokine producers in CD4+ T cell subsets, we examined if there is an effect of HLA B27 on the CD4+ TH1/TH2 commitment in HLA B27-positive individuals. To this end we analysed the degree of in vivo polarization in CD4+ T cell compartments differentiated by the surface expression of CD45RA and CD27. Though there was a significantly different coexpression of IFNγ and IL-4 in CD27- memory T cells as compared to differentiated memory CD4+ T cells, no differences between HLA B27-positive and negative donors were observed. These findings argue against a general defect or perturbation of TH1/TH2 differentiation in HLA B27 positive individuals despite a lower secretion of proinflammatory cytokines TNFα and IFNγ in antigen-experienced CD8+ T-cells.

Altogether, a detailed analysis of CD4+ T cell characteristics, cytokine production and TH1/TH2 differentiation did not reveal any significant differences between HLA B27-negative and -positive healthy individuals and AS patients. On the other hand, our study revealed a marked defect in proinflammatory cytokine secretion restricted to memory and effector CD8+ T cells of HLA B27-positive AS patients and healthy controls. These findings point towards an important influence of HLA B27 on the inflammatory cytokine production of antigen-experienced CD8+ T cells.
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