The involvement of NK cells in ankylosing spondylitis

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Abstract

A role for NK cells in the regulation of autoimmunity has been demonstrated. Since there is a strong association between Ankylosing Spondylitis (AS) and HLA-B27, which is specifically recognized by the NK-inhibitory receptor KIR3DL1, this study evaluated the potential involvement of NK cells in AS. We studied 19 AS patients and 22 healthy volunteer donors and assessed the percentage, activity and receptor expression of peripheral blood NK cells. We also evaluated candidate-inflammatory mediators in sera. We found that AS patients have significantly higher percentages of NK cells. However, we found no differences between the ability of NK cells derived from AS and healthy controls to recognize target cells expressing HLA-B27. Remarkably, we observed that the NK-inhibitory receptor CEACAM1 (carcino-embryonic antigen-cell adhesion molecule) is highly expressed among AS-derived NK cells. Furthermore, engagement of CEACAM1 inhibited NK activity in these patients. Finally, we demonstrated that CEACAM1 expression is induced by IL-8 and SDF-1 (stromal cell derived factor), both of which are present in high levels in the sera of AS patients. These results may indicate that NK cells and CEACAM1 play a role in AS pathogenesis and implicate chemokines in the mechanism of CEACAM1 expression.

Introduction

NK cells have a crucial role in the initial defense against viral infections and transformed cells. They quickly respond by killing the abnormal cells and by releasing immunomodulatory cytokines such as IFN-γ. Human NK cells are identified by the expression of CD56 and lack of the CD3 complex and comprise 5–10% of the PBMCs. The killing activity of human NK cells is balanced by contra-regulatory signals derived from inhibitory and activating receptors (1). The NK-inhibitory receptors mostly include the Killer Ig-like Receptor (KIR) family that mainly recognizes MHC class I molecules (2). In addition, we have recently identified a MHC class I-independent inhibitory mechanism of NK cell cytotoxicity that is mediated via homophilic CEACAM1 interactions (3–5). The activating NK receptors include CD16 (6) and the natural cytotoxicity receptor (NCR) family composed mainly of NKP30 (7), NKP44 (8) and NKP46 (9).

NK cells also play a key role in regulating autoimmune responses (10). Decreased numbers and impaired function of peripheral blood NK cells in patients with autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, Sjogren’s syndrome, Rheumatoid Arthritis (RA) and type I diabetes were documented by several studies (10–12). However, NK cell lymphocytosis and leukemia are also associated with autoimmune syndromes, such as vasculitis and RA (13–16). Alterations in KIR repertoire expression on NK cells and on T cells have also been associated with autoimmune diseases such as Behcet’s disease, type I diabetes and psoriasis (17–19).

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In this study, we focus on the involvement of NK cells in ankylosing spondylitis (AS). AS is a complex and debilitating disease with a worldwide prevalence ranging up to 0.9% (20). The etiology and pathogenesis of AS are not yet fully understood and diagnosis is difficult. The HLA-B27 gene is present in ~90–95% of Caucasian patients with AS in Central Europe and North America (20). However, it seems that additional genes outside the HLA region are also involved in AS (21). Indeed, linkage to other non-MHC sites was noted, specifically to loci on chromosomes 1, 2, 9, 10, 16 and 19 (22).

Methods

Patients and healthy donors
An institutional review board approved these studies and informed consent was provided according to the Declaration of Helsinki. After informed consent, blood was obtained from patients (AS01–AS19, aged 25–58, Table 1) who satisfied the diagnostic criteria of the American College of Rheumatology for AS (18), from 22 healthy control donors (aged 20–65) and from 14 patients with anterior uveitis (aged 21–74), 7 of whom expressed HLA-B27. The severity of the disease was measured by the Bath Ankylosing Spondylitis Functional Index (23) and the Bath Ankylosing Spondylitis Activity Index (24) (Table 1). All patients received similar treatment with non-steroidal anti-inflammatory drugs and/or cyclooxygenase-2 inhibitors. Two of the patients received salazopyrin and four patients received no treatment. None of the patients received tumor necrosis factor (TNF)-α inhibitors. PBMCs were separated and the plasma was harvested and stored at −20°C until analysis. PBMCs were isolated from the cell pellet using Ficoll density gradient.

Isolation of peripheral blood NK cells
NK cells were purified from PBMCs using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec Inc., Bergisch-Gladbach, Germany) according to the manufacturer instructions. NK cells that stained positive for CD56 and negative for CD3 were either used immediately or cloned in the presence of hrIL-2 and grown in culture as previously described (25).

Cell lines and antibodies
The cell lines used in this work were the MHC class I-negative human EBV-transformed B cell line 721.221 (221). The generation of .221 transfectants expressing CEACAM1 (.221/CEACAM1), HLA-Cw3 (.221/Cw3), HLA-Cw4 (.221/Cw4), HLA-Cw6 (.221/Cw6), HLA-G (.221/G) and HLA-B27 (.221/B27) proteins was previously described (3, 25, 26). Cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS (heat inactivated), 1 mM L-glutamine, 1 mM penicillin–streptomycin, 1 mM non-essential amino acids and 1 mM sodium pyruvate (GIBCO BRL, Gaithersburg, MD, USA). Polyclonal antibodies used in this work were the rabbit anti-human CEACAM1, CEACAM5 and CEACAM6 antibodies (DAKO). The control rabbit anti-ubiquitin antibodies were a kind gift of Prof. Eitan Yefenof. mAbs used in this work were anti-CD66 a,b,c,e Kat4c (DAKO) and the anti-CD99 mAb 12E7. The production and the specificity of the NKp46 antiserum were previously described (27).

Table 1. Clinical characteristics of AS patients

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<tr>
<th>Patients</th>
<th>Age</th>
<th>Sex</th>
<th>HLA</th>
<th>BASFI</th>
<th>BASDAI</th>
<th>Disease durationa</th>
<th>Peripheral jointsb</th>
<th>Chronic diseases</th>
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<td>M</td>
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<td>M</td>
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</table>

Patients were arranged according to the expression of HLA-B27. BASFI, Bath Ankylosing Spondylitis Functional Index; BASDAI, Bath Ankylosing Spondylitis Activity Index.

aDisease duration refers to the years since diagnosis. bIn the Peripheral joints column 'yes' indicates inflammation of peripheral joints.
Human PBMCs were resuspended at 1 x 10^6 cells ml⁻¹ in RPMI, 10% human serum supplemented with 1 mM glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate and 1 mM Penicillin Streptomycin (all from Gibco BRL) in six-well plates without or with the recombinant cytokines IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α, SDF-1 (Pepro Tech EC, London, UK) at the indicated concentrations for 48 h at 37°C in 5% CO₂.

**Statistical analysis**

The mean cytokine plasma concentrations of patients with AS and the mean concentrations of control donors were compared using the one-tail Student’s t test assuming equal variances. Relationships between the level of cytokine protein and other parameters in AS patients were both analyzed with the Pearson correlation tests.

**Results**

**AS patients have increased proportion of NK cells in peripheral blood**

A clear association exists between the development of AS and the presence of HLA-B27. Since HLA-B27 protein is specifically recognized by the NK-inhibitory receptor KIR3DL1 (29), we investigated whether NK cells might play a role in AS.

To evaluate the proportion of peripheral blood NK cells, PBMCs were isolated from normal subjects and AS patients and double stained for CD3 and CD56 expression. Remarkably, a significantly higher percentage of NK cells were observed in AS patients as compared with the healthy individuals (mean value 12.7% versus 6.4%; P < 7 x 10⁻⁶) (Fig. 1A). Importantly, even in two healthy controls who expressed HLA-B27, the NK cell levels were similar to the levels of other healthy individuals (Fig. 1A). The normal range of NK cell percentages in dozens of other normal subjects tested in our lab (data not shown) has always been between 5 and 10%, similar to the data shown here. However, the range of NK percentages in AS individuals reached up to 24%. In addition, we found no contribution of the age, sex and treatment factors to the differences in the NK percentages and all other tested parameters.

We next investigated whether the increased number of NK cells in AS patients represent a specific enrichment of certain NK cell populations capable of interacting specifically with HLA-B27. We tested 39 NK clones derived from AS patient 02.
CEACAM1 expression is elevated in AS patients and is induced by AS sera

To further characterize the NK cell population in the AS patients, we examined the expression of various receptors on the bulk NK population. No significant differences were observed in the expression of CD16, CD62L, CD53, CD7, KIR3DL1, Nkp46, CD48, CD69, CD95 and CD100 (data not shown). It should be noted that the expression of CD25, the high-affinity alpha subunit of IL-2R, was up-regulated in some of the AS patients (AS 06, AS 01 and AS 13). This observation suggests that in some of the AS patients the NK cells are found in an activated state.

Our group identified and characterized in the past few years a novel MHC class I-independent inhibitory mechanism that is mediated via the CEACAM1 homophilic interactions (3). In the past, expression of CEACAM1 was shown to be significantly elevated only after IL-2 activation of NK cells derived from peripheral blood or decidual, and of activated NK cells derived from transporter associated with antigen processing (TAP)-2-deficient patients (3–5). We therefore examined the expression of CEACAM1 on freshly isolated NK cells. Strikingly, significant expression of CEACAM1 was observed on fresh NK cells derived from AS patients as compared with healthy donors (mean 12.57% versus 1.06% for AS and normal donors, respectively, P < 0.00007, Fig. 2A). Moreover, expression of CEACAM1 was also low in the two healthy individuals who expressed HLA-B27 (Fig. 2A). Remarkably, in one AS patient (AS 06), the expression of CEACAM1 was detected on almost all NK cells (Fig. 2A). Even when ignoring patient AS 06 (because of the unusual expression of CEACAM1), still, a mean of 7.67% of the NK cells derived from the AS patients express CEACAM1. This mean is significantly higher than the mean of 1.065% of the NK cells derived from the healthy donors. It is noteworthy that there was no correlation between the percentage of NK cells expressing CEACAM1 and the total number of NK cells in each patient.

The factors that are responsible for the up-regulation of CEACAM1 on NK cells are still largely unknown. It seemed conceivable that factors in the patients’ sera might be involved. We therefore incubated fresh PBMCs derived from a healthy individual with sera derived either from AS patients or from healthy controls. PBMCs were analyzed by triple staining to evaluate CEACAM1 expression on gated NK cells. Importantly, up-regulation of CEACAM1 expression was observed only when PBMCs were incubated with the AS patients sera (Fig. 2B). Furthermore, the ability of each serum to induce CEACAM1 expression on healthy NK cells correlated with the CEACAM1-positive NK cell percentage observed in that particular serum donor. Hence, the sera of AS patients who did not have a predominant CEACAM1-positive NK percentage did not induce CEACAM1 expression, similar to the sera of healthy donors. In contrast, the sera of AS patients who showed larger percentages of CEACAM1-positive NK cells did induce a significant expression of CEACAM1 on healthy NK cells (Fig. 2B).

There is a strong positive correlation of 98% between the level of CEACAM1 expressed on the NK cells derived from the serum donors and the level of CEACAM1 expressed on the
healthy NK cells after incubation with sera (86% if we exclude the exceptional observation of AS06). Linear regression analysis shows an $R^2$ of 97.8% and a $P$-value of 0.000022. An exponential regression analysis shows an $R^2$ of 98.4% and a $P$-value of 0.00001. These facts suggest that the level of CEACAM1 in the donor’s sera affects the expression of CEACAM1 in the healthy NK in an exponential manner.

Elevated levels of SDF-1 and IL-8 in the sera of AS patients induce up-regulation of CEACAM1

To identify the factors that are present in the sera of the AS patients and that are involved in the up-regulation of CEACAM1 we used ELISA. The levels of four different cytokines were determined, including TNF-α, GM-CSF, IFN-γ and IL-2. However, no statistically significant differences between the patients’ sera and the healthy donors could be observed (data not shown).

We next tested whether chemokines would be over-expressed in the sera of AS patients. Strikingly, AS patients exhibited significantly increased serum levels of IL-8 and SDF-1 as compared with controls (Fig. 3A and B, respectively). Importantly, a significant correlation was observed between IL-8 levels and the percentage of NK cells expressing CEACAM1 (Fig. 3C), suggesting that this cytokine might be implicated in the expression of CEACAM1 on the surface of NK cells. Additionally, regression analysis indicated a significant positive linear relationship between the duration of illness and the SDF-1 concentration (Fig. 3D). Thus the SDF-1 levels might be used as a clinical parameter to try and estimate the duration of illness.

Our next goal was to study whether the elevated levels of IL-8 and SDF-1 in the AS patients could induce CEACAM1 expression. PBMCs derived from healthy individuals were cultured either in the presence of IL-8 and SDF-1 or with a cytokine-free medium (Fig. 4A) for 48 h. IL-8 and SDF-1 were used either in the physiological concentration found in healthy donors (Fig. 4B) or in the higher average concentrations found in AS patients (Fig. 4C). Strikingly, incubation of PBMCs with the average concentrations of IL-8 and SDF-1 found in AS patients’ sera induced the expression of CEACAM1 on a significant proportion of NK cells (Fig. 4C).

Interestingly, two sub-populations of NK cells expressing CEACAM1 at high and low levels could be observed (Fig. 4B). In contrast, there was no significant expression of CEACAM1 when either no chemokine was present (Fig. 4A) or when NK cells were incubated with the chemokines in the level found in healthy donors’ sera (Fig. 4B).

CEACAM1 is a functional inhibitory receptor on AS patients’ NK clones

The function of the CEACAM1 on AS NK cells was assessed in NK killing assays. NK cells were isolated from PBMCs of various donors and subsequently cloned and grown in the presence of IL-2. To demonstrate that the CEACAM1 expressed on NK cells from AS patients is functional, various NK clones positive or negative for CEACAM1 were tested in redirected killing assays. In this type of assay, the killing of the murine P815 mastocytoma cells is induced by anti-NCR mAbs that are bound to the P815 cells with their Fc-portion and activate killing by the cross-linking of the NCR. As expected, the killing of P815 cells was induced by pre-incubation of the P815 cells with anti-NKp46 mAb (Fig. 5A). The addition of anti-CEACAM mAb (Kat4c) inhibited the redirected killing of P815 by the CEACAM1-positive NK clones only (Fig. 5A). The control anti-CD99 mAb (12E7) had little or no effect (Fig. 5A).

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**Fig. 3.** Elevated levels of IL-8 and SDF-1 concentrations in sera of AS patients. AS patients exhibited significantly higher serum levels of both chemokines IL-8 (A) and SDF-1 (B) compared with controls as measured by ELISA. Regression analysis indicated a significant linear relationship between IL-8 levels and the percentage of NK cells expressing CEACAM1 (C) and between the duration of illness and the SDF-1 serum concentration (D). Pearson $r$-values and $P$-values are specified in the upper right of the relevant panels.
CEACAM1 interacts homophilically to confer protection. The functional significance of CEACAM1 expression was therefore also assayed in killing assays against .221 cells and .221 cells expressing CEACAM1 (.221/CEACAM1) (3). The killing activity of AS-derived CEACAM1-positive NK clones was inhibited only when .221/CEACAM1 cells were used as targets (see representative clone in Fig. 5B). This inhibition was the result of CEACAM1 homophilic interactions, as lysis was restored when anti-CEACAM F(ab')2 antibodies were included in the assay (Fig. 5B). The control anti-ubiquitin antibodies had little or no effect (Fig. 5B). No inhibition was observed when AS-derived CEACAM1-negative NK clones were used (Fig. 5C). These combined results demonstrate that the CEACAM1 protein functionally inhibits NK killing activity in AS patients.

Discussion

The term spondyloarthropathy (SpA) describes and defines a group of related inflammatory joint diseases and a tendency to involve the sacroiliac joints (30). Five subgroups were defined: AS, reactive arthritis/Reiter’s syndrome, psoriatic arthritis, arthritis associated with inflammatory bowel disease and undifferentiated SpA. The pathogenesis of SpAs is still unknown, but an inflammatory infiltrate composed of lymphocytes, plasma cells and, sometimes, of polymorphonuclear cells (PMNs) is observed in the inflamed joints. This inflammatory process is erosive and is followed by fibrous tissue proliferation, leading to the formation of cartilage and, subsequently, of bone (31).

Our results demonstrate that compared with healthy controls, AS patients have a significantly higher percentage of NK cells. Moreover, a prominent increase in CEACAM1 expression has been demonstrated among AS-derived NK cells.

CEACAM1 is an Ig superfamily-related glycoprotein with homologues in rodents (mice and rats) and humans. It is widely expressed on a variety of epithelial cells and hematopoietic cell types including PMNs, NK cells, B cells, monocytes and dendritic cells (32). The function of CEACAM1 seems to be cell-type specific. For example, ligation of CEACAM1 on PMNs enhances the respiratory burst and up-regulates the integrin adhesion function (33). In contrast, CEACAM1 expression on activated human T cells, NK and decidual NKT cells is associated with the inhibition of activity (3, 5, 34).

Homophilic CEACAM1 interactions transduce the inhibitory signals through the cytosolic ITIM sequences (3). Human CEACAM1 can interact heterophilically with other CEACAM members, such as CEACAM5 (35), E-selectin (through expression of the sialyl-Lewis antigen by CEACAM1) and various cell surface structures of bacteria, such as the fimbria of Escherichia coli and Salmonella (35). Therefore, it is noteworthy that an immune response against an unknown auto-antigen is believed to play a crucial role in the pathogenesis of AS and pathogens are thought to be involved in the initiation of this reaction (36). Several candidate pathogens have been suggested, however, not all received confirmation from experimental data (36).

The best-known ligand for CEACAM1 is CEACAM1 itself. Characterization of the CEACAM1 expression in the sacroiliac joints of AS patients is therefore of major importance; however, at present, due to ethical issues and the relative inaccessibility of this tissue, it is almost impossible to examine this tissue directly. In addition, AS patients who develop arthritis of accessible peripheral joints are rare; nevertheless, a few of these patients have been investigated and the expression of CEACAM1 on PMNs of RA and SpA patients was found to
be increased by 4-fold in the synovial fluid, compared with peripheral blood PMNs of the same patients (37).

Confirming our results, a recent study by Dalbeth et al. (30) reported that NK cells composed 16.1% (±SD 9.3%) of all lymphocytes within the synovial fluid of RA and two SpA patients. Similar percentages of NK cells were found in PBMCs derived from these patients (30). Moreover, the majority of NK cells in the synovial of these two patients expressed high levels of CD56 and did not express CD16 or KIR/killer activatory receptors (KAR) (30). The CEACAM1 expression was not investigated in these patients; however, we and others demonstrated that expression of CEACAM1 protein is mostly limited to the surface of activated, CD16-negative CD56 bright NK cells (3).

Other studies have attempted to determine the cytokines involved in the pathogenesis of AS. Contradicting results were obtained: pro-inflammatory cytokines, particularly TNF-α, were observed in some SpA (38). However, we and others failed to reproduce these results (38–40) and even found a significant increase in serum anti-inflammatory cytokines (38, 41). Here we demonstrate [in agreement with other studies (30, 38, 41–43)], that the sera of AS patients contain higher levels of chemokines, such as IL-8 and SDF-1. Importantly, it has been established that serum IL-8 levels correlate with acute phase reactants such as C-reactive protein and haptoglobin, suggesting that serum IL-8 may reflect clinical activity of the disease (42). In addition, the presence of IL-2, IL-8 and SDF-1 was demonstrated in the arthritic joints of SpA patients (43–46).

This led us to investigate whether these chemokines control the expression of CEACAM1. Indeed, here we show for the first time that the combination of IL-8 and SDF-1 results in the up-regulation of CEACAM1 expression on a sub-population of NK cells. Strikingly, in a recent study, the CXCR4 receptor, which is the specific receptor for SDF-1, was found to be highly expressed in SpA patients as compared with normal subjects (43).

Support for the fundamental role of CEACAM1 in the pathogenesis of AS comes from genetic studies. The carcino-embryonic antigens (CEA) family genes are clustered on human chromosome 19q13.2 (47). Laval et al. performed a genome-wide scan on 185 families containing 255 sib pairs with AS. In their study, although the MHC locus was identified as encoding the greatest component of susceptibility with an overall logarithmic odds (LOD) score of 15.6, their results strongly supported the presence of non-MHC genetic susceptibility factors in AS. Suggestive linkage was observed with the marker D19S420 (LOD 3.58), which is located on chromosome 19q13.2. As mentioned above, this is the genomic location of CEACAM1 (22).

A central question that remains to be addressed regards the specificity of these findings to AS. Acute anterior uveitis (iritis) is frequently the first indication of a formerly undiagnosed HLA-B27-associated extra-ocular disease. The most common of these diseases are SpAs (48). About two-thirds of patients who have HLA-B27-associated uveitis present to the ophthalmologist without a diagnosis of inflammatory joint disease. Approximately 50% of patients with acute anterior uveitis have HLA-B27 (49, 50) and up to 90% of these patients also have SpA (50). Indeed, we found a significant elevation in CEACAM1 expression on fresh NK cells derived from four HLA-B27-related uveitis patients out of seven tested, as compared with only one patient with uveitis not related to HLA-B27 out of seven tested (data not shown).

Finally, it seems that CEACAM1 expression is in some way connected to either the loss of MHC class I or to the presence of specific MHC class I proteins. A dramatic up-regulation of CEACAM1 expression was observed in class I-deficient melanoma cells (3), in cells derived from TAP-2-deficient patients (4) and in trophoblast cells expressing only some of the HLA alleles (5), and as shown here, in AS patients who are mostly HLA-B27 positive.

In light of our observations, we propose a model in which NK cells are attracted to joints of AS patients by IL-8 and SDF-1. NK cells enter the highly vascular AS synovial membrane, and upon arrival at the synovium, in the presence of IL-2, IL-8 and SDF-1, a subset of NK cells expressing CEACAM1 is
significantly expanded. These NK cells bind to CEACAM1 found on the surface of synoviocytes and PMNs, and their cytolytic activity is inhibited. In this scenario, NK cells may contribute to the pathogenesis of AS by not being able to kill the autoimmune cells that might be causing the disease. However, it is also possible that inhibited NK cells are restrained from eliminating an antigen derived from a yet unknown pathogen responsible for the disease symptoms. This presumed pathogen may also bind to CEACAM1 and directly inhibit NK cells activity, similar to the putative CEACAM1 ligand induced upon infection with CMV (5).

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Abbreviations

AS ankylosing spondylitis
CEA carcino-embryonic antigens
CEACAM carcino-embryonic antigen-cell adhesion molecule
FBS fetal bovine serum
GM-CSF granulocyte macrophage colony-stimulating factor
KAP killer activatory receptors
KIR killer Ig-like receptor
L0D logarthmic odds
NCR natural cytotoxicity receptor
PMNs polymorphonuclear cells
RA rheumatoid arthritis
SDF stromal cell derived factor
SpA spondyloarthropathy
TAP transporter associated with antigen processing
TNF tumor necrosis factor

References


