Phenotypic Study of Natural Killer Cell Subsets in Ankylosing Spondylitis Patients

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ABSTRACT

It has been demonstrated that natural killer (NK) cells play a role in regulation of autoimmunity. They play a protective role in several rodent disease models. In this study we aimed to compare the immunophenotypic features of NK cells in Ankylosing Spondylitis (AS) with normal subjects with regard to CD56 and CD16 molecules.

This study was carried out on 30 AS patients and 33 normal volunteer donors. Peripheral Blood Mononuclear cells (PBMC) were tested by flow cytometry detecting the intensity of CD56 and CD16 surface molecules. The percentage of positive cells and their subsets were then calculated and statistically analyzed using SPSS software. A significant increase was found in CD56⁺ CD16⁺ (P ≤ 0.009), and also in the subset of CD56[^dim] CD16⁺ (P ≤ 0.02), but not in CD56[^bright] CD16⁺ (P=0.3) NK cells in AS patients compared to controls. We conclude that these results may indicate that NK and their subset ratios play a role in AS pathogenesis. Moreover, determination of NK subsets in combination with clinical features may be useful for AS diagnosis. However, further studies using large samples together with determination of relevant cytokines are recommended to verify the exact role of NK in AS disease.

Key words: Ankylosing Spondylitis; CD56; CD16; Natural Killer cell

INTRODUCTION

Ankylosing spondylitis (AS) is an inflammatory disease characterized by ankylosis of the joints. AS is a young adulthood disease with prevalence of approximately 0.1–0.8% in Caucasians.¹ The patients usually suffer from chronic pain and significant disability. A strong association has been demonstrated between AS and HLA-B27.²

Natural killer (NK) cells principally contribute to innate immunity and adaptive immune responses by killing target cells and production of a variety of cytokines and chemokines. They may also play a role in the regulation of autoimmunity.³ ⁴ The number and
function of NK cells decline in several autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, Sjogren’s syndrome and Rheumatoid Arthritis.\(^5\,^6\) Moreover, NK cell lymphocytosis and leukemia have been reported in vasculitis and RA. Although several association studies in spondyloarthopathies (SpA) and also its relationship with HLA-B27 have been reported,\(^7\,^11\) few studies have experimentally been conducted concerning the pattern of NK subpopulations in AS so far.

Different NK cell subpopulations can be determined according to the presence and density of CD56 and CD16 (Fc gamma receptor III) surface molecules.\(^1\,^2\) NK cells, neurons, glia and , skeletal muscle express CD56 (Neural Cell Adhesion) Molecule on their surfaces. CD56 contributes in cell-cell adhesion.\(^13\) Low density CD56 (CD56\(^{\text{dim}}\) ) subsets are more than 90% of peripheral blood NK cells which express perforin and killer immunoglobulin like receptors. CD16 has been identified as Fc receptors binding to the Fc portion of IgG antibodies. This receptor is expressed on the surface of NK, neutrophil, monocyte and macrophage.\(^14\) A subpopulation of CD56 dim NK cells expressing CD16 marker is involved in antibody-dependent cellular cytotoxicity (ADCC). The subset of CD56 bright NK cells, which are rare in blood but are the predominant cell type in lymph nodes and tissues do not express Perforin and KIRs. It has been hypothesized that the CD56 bright subset exhibits immunoregulatory functions through the secretion of various cytokines (i.e. IFN-γ, TNF-α, or IL-10), whereas CD56 dim cells are responsible for the cytolytic activity against virally infected cells and autologous tumor cells.\(^15\)

Due to the involvement of NK cells in AS and different autoimmune diseases, the aim of this study was to assess the presence and intensity of CD56 and CD16 markers on the surface of NK cells of AS patients in comparison with normal controls in order to verify the involvement of NK subsets in this disease. We also aimed to indicate whether the NK subsets have been altered in peripheral blood of AS patients.

**PATIENTS AND METHODS**

Subjects- A total of 33 AS patients and 35 normal donors took part in this study. An institutional review board approved this study and informed consent was provided according to the Declaration of Helsinki. Patients who satisfied the diagnostic criteria for ankylosing spondylitis proposed by van der Linden for modification of the New York criteria\(^16\) were considered in the study. Peripheral blood was obtained from patients and normal donors aged 20–55 years.

The severity of the disease had been measured by the Bath Ankylosing Spondylitis Functional Index\(^17\) and the Bath Ankylosing Spondylitis Activity Index.\(^18\) The mean values for BASFI and BASDI were 3.5±1.8 and 4.0±2.1 respectively. Rheumatoid factor was negative and the patients did not show immunological abnormalities (serum immunoglobulin and complement were within normal values). All patients received similar treatments with non-steroidal anti-inflammatory and/or cyclooxygenase-2 inhibitors.

Isolation of peripheral blood mononuclear cells (PBMCs) - Peripheral venous blood samples were collected into tubes containing heparin, PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque Plus solution (Amersham Bioscience, Sweden). Each PBMC was incubated in a culture dish in a humidified 5% CO\(_2\), and 95% air atmosphere at 37°C for 60 minutes. After incubation, nonadherent cells were collected and the cell suspensions were washed twice in phosphate-buffered saline (PBS).

Sample preparation- cells were then resuspended in ice-cold staining buffer (PBS, 3% FCS) at 5× 10\(^6\) cells/ml and cells were double stained in aliquots of 100 μL with fluorochorome- conjugated monoclonal antibodies for 20 mints. Anti human CD16 Ab (VEP13) conjugated with phycoerythrin (Milenyi Biotec, Germany) and monoclonal Ab to human CD56 antigen conjugated with fluorescein isothiocyanate (FITC) from Invitrogen (Canada) were applied for cell staining according to manufacturer procedures. Cells were then washed once by centrifugating at 540 g and resuspended in 0.2 M of PBS for introduction in the flow cytometer.

Flow cytometry- Data acquisition was carried out in a Partec flow cytometer. Lymphocytes were gated into the R1 window on size versus granularity dot plots. In all experiments at least 30 000 events were counted and the results were expressed as the percentage of cells in a gated lymphocyte region. CD56\(^{-}/\)CD16\(^{+}\) NK cells and their subsets (according to the intensity of CD56 molecule) were selected and results were expressed as the percentage of cell subsets within the lymphocyte population (as depicted Figure 1). For each antigenic determinant, the percentage of positive cells stained above the negative control values (unstained cells) was...
Figure 1. Phenotypic profile of PBMCs from AS patients and healthy donors. Peripheral mononuclear cells were absorbed for adherent cells, stained with CD56- FITC and CD16- PE mAbs. Cells were gated on lymphocytes for each experiment. Representative dot plots illustrate the distinct immunophenotypic patterns for each NK subtypes. (Q2 = the percentage of CD56/CD16, QA4/Q4 = CD56\textsubscript{dim}/CD16\textsuperscript{+} and QA2 = CD56\textsubscript{bright}/CD16\textsuperscript{-} populations)

determined. The proportion of CD56\textsuperscript{+} CD16\textsuperscript{+}, CD56\textsubscript{dim} CD16\textsuperscript{+} and CD56\textsubscript{bright} CD16\textsuperscript{-} subpopulations were calculated using dot plots.

Statistical Analysis
To establish the statistical significance of the immunophenotypic differences observed between CD56\textsubscript{dim} and CD56\textsubscript{bright} NK cells data were analyzed using a Student's t-test or Mann-Whitney test, with 95% confidence. P values less than 0.05 were considered to be associated with statistically significant differences.

RESULTS
In this study, since the adherent cells were excluded from PBMCs, and because of the lack of CD16 on T cells, all the CD56/CD16 positive subpopulations gated in lymphocytes were considered as NK cells.

Figure 2. Comparison of NK subsets in AS and Healthy subjects. Non adherent Peripheral blood mononuclear cells were stained with CD56-FITC and CD16-PE mAbs. Lymphocyte subsets were then determined by flow cytometry. Results from CD56\textsuperscript{-} CD16\textsuperscript{+} and CD56\textsubscript{dim} CD16\textsuperscript{+} subsets in two groups showed statistically significant differences (p< 0.009 and p< 0.02 respectively).
Using dot plots from flow cytometry, we calculated the percentage of each CD56\(^+\)/CD16\(^+\), CD56\(^{dim}\) CD16\(^+\) and CD56\(^{bright}\) CD16\(^+\) subpopulations within the gated lymphocytes.

The comparison of these subsets between AS and normal subjects indicated significant elevations of CD56\(^+\) /CD16\(^+\) NK cells (P<0.009) and CD56\(^{dim}\) /CD16\(^+\) subsets (P<0.02) in AS patients. We also noticed a higher, but not statistically significant (P=0.3) increase of CD56\(^{bright}\) CD16\(^+\) subset in AS comparing with normal controls.

**DISCUSSION**

In this study we evaluated the involvement of NK subsets in AS. We assessed the percentages of NK cell subsets in peripheral blood from 30 AS patients and 33 normal volunteers in order to identify the differences in NK phenotypes. NK cells may drive, shape, and regulate the activities of other immune cells and affect adaptive immune responses. The ability to respond to a variety of cytokines suggests that the local microenvironment in which NK cells exist may shape and modulate their function.\(^\text{19}\) CD56\(^{dim}\) NK cells are often regarded as being specialized in cytotoxicity acting in the periphery, while CD56\(^{bright}\) NK cells are considered specialized in the secretion of cytokines and chemokines acting in secondary lymphoid organs and at sites of inflammation.\(^\text{20}\) Moreover, CD56\(^{bright}\) NK cells can trigger differentiation of monocytes into dendritic cells.\(^\text{21}\) Such positive feedback loops can well promote the pathogenesis of chronic inflammatory conditions.\(^\text{22}\)

Associations between NK cell activity and autoimmune conditions have been mentioned in several reports, including studies with finding altered numbers, phenotypes, and functions of NK cells.\(^\text{23,24}\) With the exception of genetic linkage analysis, relatively few studies have been undertaken to explore the role of NK cells in human autoimmunity. Recently, Shamji et al reviewed the current literature on the origin and pathophysiology of AS, focusing on genetic and molecular associations, consequent pathomechanisms, and associated triggers. They discussed that understanding of the initiating molecules and genetic factors of AS is helpful for specific treatment of this disease.\(^\text{25}\) It is suggested that NK cells and their receptors could play a role in AS development. Szántó S. et al reported that the frequencies of CD4\(^{+}\) T helper and CD56\(^{+}\) NK cells were higher in peripheral blood of AS patients. Their results provided evidence for an altered cell subset distribution and intracytoplasmic cytokine balance in AS.\(^\text{26}\) Furthermore, it has previously been reported that NK cell numbers and activities were reduced in patients with systemic autoimmune diseases.\(^\text{27,28}\) This observation might help us to understand the mechanisms underlying these numerical and functional deficiencies of NK cells. One possible explanation for this is that NK cell depletion in systemic autoimmune diseases such as SLE patients is secondary to disease. Moreover, these observations indicate that NK cell apoptosis induced by circulating immune complexes and serum cytokines might contribute to NK cell depletion in some autoimmune diseases (e.g., SLE, Sjogren’s syndrome, and RA). However, in our study reduced number of NK cells and their subpopulations were not observed in patients with AS. This could be due to the absence of circulating immune complexes. Instead, our results demonstrated that compared with healthy controls, AS patients had a significantly higher percentage of CD56/CD16 double positive and CD56\(^{dim}\) CD16\(^+\) NK subset. Confirming our results, a study reported by Niva Azuz-Lieberman et al indicated that AS patients have significantly higher percentages of NK cells. However, they found that NK cells from AS patients showed no differences in ability of recognition of NK cells HLA-B27 bearing target cells with those from normal.\(^\text{29}\) Moreover, Dalbeth et al reported that NK cells composed 16.1% of all lymphocytes within the synovial fluid of RA and two SpA patients.\(^\text{30}\) They reported that the majority of synovial NK cells from SPA patients were high level presenting CD56 marker, while the percentages of NK cells in PBMCs of all patients were similar. Since there is no documented report on CD56\(^{dim}\) and CD56\(^{bright}\) NK subsets in AS so far, in the present study CD56/16 double positive NK subpopulations were evaluated in Iranian AS patients.

In general, NK cells are phenotypically characterized as CD3\(^-\)/ TCR/CD16\(^-\)/CD56\(^-\)/CD94\(^-\)/CD122\(^-\)/CD158\(^-\)/CD161\(^+\) cells. However, a number of markers such as CD158 and CD161 represent multigene families. Similar to other hematopoietic cells in humans, mice, or rats there is no unique phenotypic marker for identification of NK lineage. Indeed, not all NK cells necessarily express all of the mentioned markers.
Consequently, multiple parameters is recommended in identifying NK cells. Due to the close relationship between NK cells and T cells, a combination of NK cell associated markers and a lack of T cell markers should be used to identify NK cells. In this study, due to some instrumental limitations, a suitable flow cytometer for triple staining was not available in our laboratory and we failed to exclude CD3 positive cells from PBMCs. Instead, we absorbed CD16 bearing adherent cells from the samples and examined CD56/CD16 double positive cells within the gated lymphocytes. Therefore, considering the cell markers we concluded that T cells were not included and all CD56/CD16 positive cells examined in this study belonged to NK population.

Since most of NK cells in the peripheral blood are CD56\(^{\text{dim}}\) CD16\(^{+}\) with high cytotoxic activity,\(^{31}\) we evaluated this NK subset and found that AS patients had significantly higher percentages of CD56\(^{\text{dim}}\) CD16\(^{+}\) subset in their peripheral blood. Therefore, we suggest these changes likely indicate the enhanced NK cytotoxic activity in AS. NK cells may function as important regulatory cells during the priming phase of adaptive immune responses and as regulatory and effector cells at the sites of target organ during inflammation.

Taken together, a better comprehension of how NK cells affect different autoimmune conditions such as AS may pave the way for new immunotherapeutic approaches toward alleviating or preventing such diseases. We contemplate that augmentation of NK and its subsets in AS patients may be involved in the pathogenesis of this autoimmune disease. However, to confirm these notions further studies on the other markers as well as cell activities and cytokines are recommended.

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