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Natural killer cell and gamma delta T cell alterations in enthesitis related arthritis category of juvenile idiopathic arthritis

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ABSTRACT

Enthesitis related arthritis (ERA) is associated with increased frequency of Th17 cells and synovial fluid (SF) IL-17 levels. Natural killer (NK) and gamma delta T cells have been recently shown to produce IL-17, thus we studied the NK and gamma delta-T cells in peripheral blood (PB) of 50 ERA, 16 other JIA patients and 19 healthy controls. We have analyzed the frequency of NK (total, CD56dim, CD56bright) and gamma delta-T cells, perforin and KIR3DL1/2 expression on NK cells and IL-17 and IFN-gamma production by them using flow cytometry. ERA patients had more NK cells with reduced perforin expression and IFN-gamma production but increased KIR3DL1/2 expression and IL-17 production as compared to controls. Also IL-17 producing gamma delta-T were increased in PB of ERA patients. Paired SF samples had NK cells with reduced perforin and KIR3DL expression. Thus increased NK and gamma delta-T cells may contribute to the inflammation in ERA by producing IL-17.

1. Introduction

Juvenile idiopathic arthritis (JIA) is a disease which has been classified into seven mutually exclusive categories by the International League of Associations for Rheumatology (ILAR) to facilitate understanding of their pathogenesis [1]. Among these enthesitis related arthritis (ERA) category forms the major proportion of JIA in Asian countries including India [2]. ERA is characterized by the presence of enthesitis and arthritis. It has remarkable similarity with adult spondyloarthropathy (SpA) in its clinical features and HLA-B27 association.

In view of association with HLA B27 a role of CD8 cells was theorized but the majority of cells in the synovial fluid are Th1 and Th17 cells with increased levels of Interleukin-17 (IL-17) and other pro-inflammatory cytokines [3]. Recently innate immune cells like natural killer (NK) cells, γδ-T cells have been shown to produce IL-17 and may contribute to disease pathogenesis [4,5].

NK cells are large granular cytotoxic lymphocytes that play a critical role in providing first line defense against tumor and viral infections. Human NK cells comprise two major subsets: $CD56^{dim}$ $CD16^{high}$ which are cytotoxic in nature and form 90% of the circulating NK cells and $CD56^{bright}$ $CD16^{low}$ which mainly produce cytokines and reside in secondary lymphoid tissues [6,7].

Depletion of NK cells in collagen induced arthritis (CIA) mice reduces the disease severity and prevents bone erosion [8] suggesting that NK cells play a role in inflammation in arthritis. $CD56^{bright}$ subsets of NK cells are enriched in synovial fluid of patients with rheumatoid arthritis (RA) and induce the proliferation of synovial fibroblast [9–11].

KIR3DL or three domain killer immunoglobulin-like receptors are inhibitory receptors of KIR family known to recognize HLA-B27 and B27 homodimers. Ankylosing spondylitis (AS) patients have increased frequency of KIR3DL2 expressing NK cells and CD4+ T cells [12]. These NK cells have an activated phenotype with enhanced survival and may have a pathogenic role in AS. However they did not study differential expression of KIR3DL2 on $CD56^{dim}$ and $CD56^{bright}$ cells separately.

Gamma delta T cells are a small subset of T cells that express TCR γδ chains and are mainly present in epithelial and mucosal tissues. By bridging innate and adaptive immunity they play an important role in host defense and immune regulation. In murine model of arthritis a higher number of γδ-T cell subset producing IL-17 has been reported [13,14]. The frequency of these cells correlated with disease severity [13,14]. In AS, IL-17 producing IL-23 receptor+ γδ-T cells are increased suggesting that γδ-T cells are also a source of IL-17 and may contribute to disease pathogenesis [15].

Limited data is available on the role of NK and γδ-T cells, cells in ERA [12]. Our previous study on gene expression profiling of ERA patients' peripheral blood and synovial fluid mononuclear cells (PBMCs and SFMCs) revealed the dysregulation of genes associated with innate cells [16]. Thus we studied the frequency and effector function of innate cells like NK and γδ-T cells, in children with ERA.

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2. Patients and methods

2.1. Patients and controls

Patient satisfying the International League of Association of Rheumatologists' criteria for ERA were included as study subjects [1]. In addition children with other categories of JIA were recruited as disease controls. Gender similar young adults were included as healthy controls as it was not ethical as well as difficult to get samples from healthy children.

2 ml peripheral blood (PB) in EDTA vial was collected for flow cytometry experiments. Synovial fluid (SF) was collected from ERA patients who required intra-articular steroid injection as a part of treatment. Clinical assessments such as tender joint counts and swollen joint counts were performed by a rheumatologist. The study was approved by the institutional ethics committee and children and parents gave consent for participation in the study.

2.2. Staining of cells for flow cytometry

Synovial fluid mononuclear cells (SFMCs) were isolated by using histopaque (Sigma, USA) density gradient centrifugation. PB and SFMCs were stained with anti CD56-FITC, anti CD3-APC, anti TCRγδ-FITC, anti perforin-PE antibodies, isotype controls (BD Biosciences, USA) and anti KIR3DL1/2-PE (Mylteni biotech, Germany). Cells were analyzed with Navios Beckman Coulter Flow cytometer.

CD3 − CD56 + cells were considered as NK cells. CD3 + CD56 + cells were considered as NK-T cells. On the basis of CD56 expression NK cells were divided into $CD56^{bright}$ and $CD56^{dim}$ (Supplementary Fig. 1A, 1B). Surface expression of KIR3DL1 and KIR3DL2 on NK cells and T cells was also assessed. CD3 + KIR3L1/2 + cells were determined in lymphocyte gate and CD56 + KIR3DL1/2 + cells were measured in CD56 gate as well as $CD56^{bright}$ and $CD56^{dim}$ gate (Supplementary Fig. 1D). Gamma delta T cells were determined as CD3 + TCRγδ + cells in CD3 gate.

Perforin content was measured by intracellular staining. Surface staining of blood and synovial cells was done with anti CD56-FITC. After fixation cells were permeabilized using permeabilization buffer and later stained with intracellular anti perforin-PE antibody. CD56 + Perforin + cells were measured in CD56 gate as well as in $CD56^{bright}$ and $CD56^{dim}$ gate (Supplementary Fig. 1E).

2.3. Cytokine production

We were not able to detect the cytokine producing NK and gamma delta T cells in stimulated whole blood. Thus peripheral blood mononuclear cells (PBMCs) were used to measure the IFNγ and IL-17 production by NK and gamma delta T cells in a separate set of patients and controls. PBMCs were isolated using density gradient centrifugation. 3×10^6 cells were cultured in RPMI media supplemented with 10% fetal bovine serum and 1% antibiotic. Cells were stimulated with 50 ng PMA (Sigma, USA) and 1 μg/ml Ionomycin (Sigma, USA). 10 μg/ml brefeldin A (Sigma, USA) was added as secretion inhibitor [17]. Cells were cultured in incubator for 6 h at 37 °C with 5% CO_2. After 6 h intracellular staining was done.

Cells were surface stained by anti CD56-FITC, anti CD3-APC and anti TCRγδ-FITC. Anti IFNγ PE and anti IL-17A PerCP (BD Bioscience, USA) were used as intracellular antibodies. CD56 + IL-17A + cells and CD56 + IFNγ + cells were measured in CD56 gate. CD3 + TCRγδ + IL-17A + and CD3 + TCRγδ + IFNγ + cells were measured in CD3 + TCRγδ + gate.

2.4. Statistical analysis

Frequency and level of expression using mean fluorescence intensity (MFI) are expressed as mean and standard deviation. Intergroup comparison and paired analysis between blood and synovial fluid was done using non-parametric tests. P value < 0.05 was taken as significant.

3. Results

3.1. Patients

The study included 50 ERA patients and 16 patients with other forms of JIA like Polyarticular JIA and Oligoarticular JIA as disease controls. Patients with systemic onset JIA were excluded as they are known to have NK cell dysfunction. Synovial fluid samples were available from 20 ERA patients. The median duration of disease was 48 months in ERA and 24 months in other JIA subjects (Table 1). At the time of inclusion all patients were receiving NSAID and 5 were on Methotrexate (MTX) therapy. Nineteen gender similar (17 males) young (median age 21 years (18–24)) healthy adults were also included in the study as healthy controls.

For detection of cytokine (IL-17 and IFN-gamma) production by innate cells, a separate set of 15 JIA-ERA patients with median age of 14.5 years (range: 10–24 years) and disease duration of 54 months (range: 3–192 months) were studied. 10 HC with median age of 21 years (range: 18–25) were also included.

3.2. Frequency of NK cells and NK cell subsets

Patients with JIA-ERA had increased frequency of NK cells (12.89% ± 5.65) as compared to healthy controls (9.34% ± 3.06; p = 0.019) and disease controls (8.81% ± 4.73; p = 0.01) (Fig. 1A). We have also analyzed the frequency of two different NK cell populations of CD56 cells as $CD56^{dim}$ and $CD56^{bright}$ population as they have different functions. Frequency of $CD56^{dim}$ as well as $CD56^{bright}$ NK cells were more in patients with ERA as compared to healthy controls ($CD56^{dim}$ = 12.3% ± 5.6 vs 8.90% ± 2.92; p = 0.02, $CD56^{bright}$ = 0.55% ± 0.37 vs 0.35% ± 0.25; p = 0.05) and disease controls ($CD56^{dim}$ = 8.62% ± 4.62; p = 0.01, $CD56^{bright}$ = 0.34% ± 0.18; p = 0.05) (Fig. 1B, C). The frequency of CD3 + CD56 + cells was no different between patients with ERA (2.93% ± 1.96) and healthy controls (2.42% ± 1.78).

No difference was observed in NK cell frequency between PB and SFMC of JIA-ERA patients. However synovial NK cells had a higher proportion of $CD56^{bright}$ cells (3.64% ± 2.66 vs 0.51% ± 0.36; p < 0.001) while $CD56^{dim}$ NK cells were decreased as compared to peripheral blood NK cells (6.69% ± 4.76 vs 12.32% ± 6.07; p = 0.008) (Fig. 1D–F).

3.3. Perforin expression in NK cell

Mean perforin expression in NK cells of JIA-ERA patients (56.19% ± 15.67) and disease controls (51.39% ± 18.83) was low as compared to healthy controls (82.79% ± 9.43; p < 0.001) (Fig. 2A). Similarly perforin expression in $CD56^{dim}$ NK cells of JIA-ERA patients (47.95% ± 18.8) and disease controls (50.8% ± 19.1) was less as compared to healthy controls (81% ± 11.87; p < 0.001). $CD56^{bright}$ NK cells of all groups had similar perforin expressions (Fig. 2B).

Perforin content in synovial NK cells of JIA-ERA patients was found to be further decreased as compared to PB NK cells (1.55% ± 2.5 vs 56.64% ±

Table 1
Clinical features of study population.

Demographic details	JIA-ERA (N = 50)	Disease control (N = 16)
Median age (range) in years	16 (7–21)	14 (7–26)
Median disease duration in months	48 (3–192)	24 (5–192)
Number with active arthritis	42	14
Number with sacroiliitis	20	None
Number with uveitis	02	None
Numbers with inflammatory back pain	11	01
HLA B27 positive	43	01