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Natural killer cells from psoriasis vulgaris patients have reduced levels of cytotoxicity associated degranulation and cytokine production

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ABSTRACT

Psoriasis vulgaris is a chronic inflammatory disease of the skin with a strong genetic component and immune system involvement. Although some evidence suggests that Natural Killer (NK) cells may play a part in psoriasis, their role is relatively unstudied and results are controversial. In this current study, NK cells from psoriasis patients exhibited reduced degranulation and produced lower levels of the pro-inflammatory cytokines IFN-γ and TNF-α. Further investigation found that NK cells from psoriasis patients and healthy controls expressed similar levels of activation markers, NK cell receptors and apoptosis-inducing molecules. In addition, comparable levels of several cytokines important in NK cell biology were found in the serum of psoriasis patients and healthy controls. Genotyping analysis revealed that HLA-C2, which provides a ligand for killer-cell immunoglobulin-like receptors (KIR) expressed by NK cells, was strongly associated with psoriasis susceptibility. However, no link between the KIR genes themselves and disease was found.

1. Introduction

Psoriasis vulgaris is a common chronic inflammatory condition of the skin which affects approximately 2% of the Caucasian population. While the cause of this disease remains elusive, a fundamental role for the immune system in its pathogenesis and maintenance has been established [1,2]. Natural Killer (NK) cells are large granular lymphocytes which function as part of the innate immune response [3]. Although they are best known for their roles against virally-infected and tumour cells they have also been implicated in playing a part in a number of human skin conditions such as atopic dermatitis [4,5], pemphigus vulgaris [6,7], and alopecia areata [8]. The role of NK cells in psoriasis is relatively unclear. There are relatively few reports and these abound with conflicting and contradictory results.

Studies investigating the functional responses of circulating NK cells in psoriasis patients have reported alterations in cytotoxicity and cytokine production. Some studies found NK cells from psoriasis patients displayed reduced cytotoxic responses [9,10]. This finding is not consistent, however, with other studies finding no differences in NK cell cytotoxicity in patients compared to healthy individuals [11–13]. A recent study found that production of the key inflammatory cytokine IFN-γ by NK cells was lower in patients relative to healthy donors [11], otherwise the role of direct cytokine secretion by NK cells in psoriasis remains poorly characterised. Alterations in the serum levels of several cytokines important in NK cell biology have been reported in psoriasis although again, many of these findings are inconsistent and controversial [14–33]. Similarly, while some groups have reported changes in circulating NK numbers and phenotype in psoriasis [11,34–38], the studies conducted are by no means comprehensive and findings vary.

Evidence for NK cell involvement in psoriasis is also found at the genetic level. There is a strong genetic component to psoriasis with a number of immune genes associated with disease susceptibility. Roles for the HLA class I family of molecules have long been appreciated with *HLA-C*, in particular *HLA-Cw*0602*, being of particular importance [39–46]. NK cells have an important multigene family of receptors, the killer-cell immunoglobulin-like receptors (KIR), which interact with HLA class I molecules and modulate NK cell activity [47]. Genetic associations between the KIR genes themselves with susceptibility to psoriasis have been reported [48–53] but, once again, these results are controversial, with some studies finding no links between KIR and psoriasis susceptibility [54,55].

Given the controversy in the literature regarding the role of NK cells in psoriasis, this current study was undertaken to comprehensively investigate the potential contribution of NK cells in a cohort of Irish psoriasis patients. Importantly while previous studies have focused on distinct facets of NK cells, this study aimed to generate a clearer picture by assessing three major aspects of NK cell biology – NK cell function, phenotype and KIR genotype – within a single cohort of psoriasis patients.

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2. Materials and methods

2.1. Donor cohorts

To investigate circulating NK cells in psoriasis, peripheral blood (n = 25) and serum (n = 21) from Irish psoriasis patients were collected in St. Vincent's Hospital Dublin. Patients had an age range of 23–72 (mean = 44 ± 15) and an average PASI score of 8.6 ± 4.3; 57.7% of patients were male. Patients were not receiving any systematic treatment at the time of sample collection. In addition, blood from 214 psoriasis vulgaris patients was collected to examine the role of HLA and KIR genotypes in psoriasis. Peripheral blood and serum was obtained from healthy normal controls (n = 23, age range 22–50) with informed consent. Approval for this study was obtained from the Ethics and Medical Research Committee, St. Vincent's University Hospital.

2.2. Isolation and stimulation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll density gradient centrifugation (Biosciences). Cells were either stimulated for 18 h at 37 °C and 5% CO_2 and then stained to assess NK cell function, or set up for immediate antibody staining to determine the phenotypic characteristics of NK cells. Stimulations were performed using 5×10^6 cells/ml in 10% foetal calf serum 1% penicillin streptomycin RPMI 1640 + GlutaMAX media (Biosciences). Up-regulation of activation markers was driven by stimulating cells with IL-15 (100 ng/ml) (Miltenyi Biotec). For the degranulation assay cells were stimulated with IFN-α (1000 U/ml) (Miltenyi Biotec) with target cells (K562 or 721.211) added to the wells for the last 4 h of stimulation at an E:T ratio of 1:2.5. For the intracellular cytokine assays, cells were stimulated with IL-15 (100 ng/ml) and IL-12 (30 ng/ml) (Miltenyi Biotec). Phosphate-buffered saline (PBS) was a negative control throughout. For the final 3 h of stimulation GolgiStop (BD Biosciences) was added to the degranulation assay wells and Brefeldin A (eBioscience) was added to the wells which were to undergo intracellular cytokine staining as per the manufacturer's instructions.

2.3. Flow cytometry

The following antibodies were used in this study anti-CD56-FITC, anti-CD56-PE, anti-CD3-PerCP, anti-CD25-FITC, anti-CD69-FITC, anti-CD107a-FITC, anti-IFN-γ-FITC, anti-HLA-DR-PE, anti-NKG2D-PE, anti-CD94-FITC (BD Biosciences), anti-NKG2A-PE, anti-LILRB1-PE, anti-NKp30-PE, anti-NKp44-PE (Beckman Coulter), anti-NKG2C-PE, anti-TRAIL-PE (R&D Systems), anti-TNF-α-PE (Biolegend) and anti-FAS ligand (AbDSerotec). For CD107a staining, the CD107a-FITC antibody (BD Biosciences) was added to cells for the final 4 h of stimulation. After the extracellular staining was complete, intracellular cytokine staining was performed using Fixation and Permeabilization Buffers (eBioscience) as per the manufacturer's instructions. Cells were acquired on a FACS Canto (BD Biosciences) or a Cyan (Beckman Coulter) and analysed using FlowJo software (TreeStar).

2.4. Serum cytokine level assays

The levels of IL-23, IL-22 and TGF-β in the serum of patients and healthy controls were assayed by ELISA (eBioscience) as per the manufacturer's instructions. Levels of IL-15, IL-12, IFN-γ and TNF-α were assessed using a multiplex assay (MSD) as per the manufacturer's instructions.

2.5. Genotyping of KIR and KIR ligand epitopes

DNA was isolated from the whole blood of donors using the QIAamp DNA Blood Mini Kit (Qiagen). Samples were genotyped for the KIR genes and for the HLA-C1 and HLA-C2 epitopes using sequence-specific primers (Sigma-Alrich). KIR genotyping was performed as previously described [56]. HLA-C epitope genotyping [57] and *HLA-Cw*06* typing [58] was performed as previously outlined. PCR was performed using a PTC200 (Bio-RAD-MJ Research) or a Px2 (Thermo Hybaid). The PCR products were then run on gels consisting of agarose in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA) (Fisher Scientific) to which ethidium bromide (Sigma) or Nancy-520 (Sigma-Aldrich) had been added. Samples were electrophoresed at 100 V for 30 min in TAE buffer and were viewed on a UVP GelDoc-It Imaging System (MSC).

2.6. Statistical analysis

Statistical analysis was performed using Prism 5 software (GraphPad). Analysis of potential differences in gene frequencies between patients and healthy controls was performed using StatCalc software (Epi Info).

3. Results

3.1. NK cells from psoriasis patients show impaired degranulation and cytokine production

In order to better understand the role NK cells play in psoriasis, PBMC from untreated patients or healthy normal controls were isolated, stimulated with cytokines and NK cell functional responses measured by flow cytometry. Both patients and healthy controls showed comparable up-regulation of the activation markers CD25 (35.08% ± 22.07%, n = 22 vs 40.39% ± 27.83%, n = 17) and CD69 (63.26% ± 2 1.75%, n = 25 vs 71.32% ± 20.10%, n = 20) in response to IL-15 stimulation, indicating that patient NK cells could be activated to a similar degree as those from healthy donors (Fig. 1a). NK cell degranulation, a correlate of NK cell cytotoxic function [59] was lower in psoriasis patients relative to healthy controls (39.02% ± 15.84%, n = 19 vs 54.62% ± 16.54%, n = 18, $p < 0.01$) (Fig. 1b) in a CD107a degranulation assay. NK cells from psoriasis patients also displayed a reduced capacity to produce important inflammatory cytokines compared to NK cells from healthy individuals (Fig. 1c). IFN-γ production was statistically significantly lower for psoriasis patients compared to controls (7.64% ± 6.98%, n = 23 vs 21.71% ± 19.96%, n = 22, $p < 0.01$) while TNF-α production showed a similar but non-significant trend (3.09% ± 1.55%, n = 6 patients vs 8.22% ± 5.46%, n = 8 controls, $p = 0.09$), possible due to the lower donors numbers assessed.

3.2. Cytokines important in NK cell biology are found at similar levels in the serum of patients and healthy controls

The ability of NK cells to produce cytokines was impaired in psoriasis patients. The assays used to examine functional NK cell responses in this study used cytokines to drive NK cell activity so it was of interest to investigate if there were any differences in the levels of these cytokines in vivo which might lead to the observed reduced NK cell responses in vitro. Therefore the levels of several cytokines important in NK cell biology were assessed in the serum of psoriasis patients and healthy controls. This panel included both cytokines that are or can be produced by NK cells (IFN-γ, TNF-α, TGF-β and IL-22) and cytokines which can act to stimulate NK cells (IL-15, IL-12 and IL-23). Despite our findings that NK cells from psoriasis patients produced lower levels of IFN-γ and TNF-α relative to healthy controls, there were no differences in the serum levels of either of these cytokines between the donor cohorts, with low levels of these inflammatory cytokines found in both patients and healthy controls (Fig. 2). Two additional cytokines produced by NK cells which have been implicated in playing a role in psoriasis, TGF-β and IL-22, were also assessed and levels were found to similar in the serum of patients and healthy donors. Furthermore, the serum levels of cytokines important in driving NK cell activity, IL-15, IL-12 and IL-23, were also found to be unchanged in psoriasis patients and healthy controls (Fig. 2).

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