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Natural killer cells in patients with polycythemia vera

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ABSTRACT

Natural killer cells (NK) are pivotal cells of innate immunity. They are potent antileukemic cytotoxic effectors. A defect in their cytotoxicity has been described in some hematopoietic malignancies such as acute myeloid leukemia, multiple myeloma and myelodysplastic syndromes. This defect is at least partially linked to a decreased or absent expression of some activating NK cells molecules, more particularly the so-called natural cytotoxicity receptors. In the present study, we more particularly focused our attention on NK cells of polycythemia vera, a myeloproliferative disease characterized by the presence of mutated JAK2 tyrosine kinase. The polymerase chain reaction analysis of NK cells from patients showed that they expressed the mutated form of JAK2. In polycythemia vera the proportion of NK was increased compared to healthy donors. The proliferative and cytotoxic abilities of NK cells from patients were similar to healthy donors. Expression of activating or inhibitory receptors was comparable in patients and donors, with nonetheless an imbalance for the inhibitory form of the CD158a,h couple of receptors in patients. Finally, the transcriptomic profile analysis clearly identified a discriminant signature between NK cells from patients and donors that could putatively be the consequence of abnormal continuous activation of mutated JAK2.

1. Introduction

Myeloproliferative disorders (MPD) are clonal pluripotent stem cell disorders characterized by a spontaneous growth of myeloid cells in bone marrow. Among the Phildadelphia (Ph1) chromosome-negative MPD, polycythemia vera (PV) often has a favorable evolution limited to thrombotic events, although an increased incidence of secondary malignancies, development of myelofibrosis or acute leukaemia may impair life expectancy [28]. Moreover, in absence of allogenic hematopoietic stem cells transplantation, these diseases are incurable. PV is treated with phlebotomy and non specific cytotoxic drug such as hydroxyurea, or more recently, in the presence of myelofibrosis, by Janus kinase (JAK) inhibitors which do not stop the evolution of disease but improve symptoms and decrease splenomegaly.

Since malignant cells in haemopathies often have abnormal human leukocyte antigen (HLA) class I molecule expression, they should be natural targets for natural killer (NK) cells although they have developed some way to escape to innate immunity [2,26]. Knowledge on NK cell functions in MPD relies on very few studies. Early studies have shown that MPD patients have decreased cytotoxic activity of NK cells despite an increase in the absolute count of CD16+. In vitro treatment with IFN-α or IL-2 restored normal NK cytotoxicity in the study of Gersuk et al. but not in the study of Froom et al. [13,14]. Among MPD, PV is characterized by a constant acquired mutation of JAK2 affecting exon 14 and, less frequently, exon 12 [3,16]. This mutation leads to a gain of function with constitutive phosphorylation and downstream signaling via STAT3 and STAT5. JAK2 participates in the signaling of the interferon gamma

Abbreviations: NK, natural killer; PV, polycythemia vera; JAK2, janus kinase 2; MPD, myeloproliferative disorders; Ph1, phildadelphia; HLA, human leukocyte antigen; STAT, signal transducer and activator of transcription; CML, chronic myeloid leukemia; KIRs, inhibitory killer immunoglobulin-like receptors; LAMP-1, lysosomal-associated membrane protein-1; NCR, natural cytotoxicity receptors.

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receptor (IFNγ-R), which is expressed by NK cells and lead to STAT1 activation that increases the expression of interferon-stimulated genes [27]. On the one hand, defect in NK cytotoxic functions have been described in many malignancies [2,26]. On the other hand, a simple view could assess that the JAK2 gain of function could induce NK cell activation and augment cytotoxicity, thus providing an increased anti-tumoral and anti-infectious defense. Of note, since IFN-γ is also produced by NK cells, JAK2 gain of function could lead to a self-entertained autocrine loop with constitutive activation of STAT1, leading to auto-immunity and susceptibility to infections, a result that is counter-intuitive with the hypothesis of increased NK activation [23]. Moreover, it has been shown that the non-classical HLA molecule HLA-G is secreted by erythroblasts in all hematopoietic organs and that this molecules turns-off the erythropoietin receptor signaling through JAK2 and JAK2 V617F dephosphorylation in PV [21]. In addition, the HLA-G molecule can be transferred to activated NK cells by trogocytosis, leading to blockade of NK proliferation and cytotoxicity owing to its interaction with the ILt2 inhibitory receptor on other NK cells [5]. Chronic myeloid leukemia (CML) is a MPD characterized by a gain of function due to constitutive tyrosine-kinase properties of the bcr-abl protein. In this disease, decreased NK cytotoxicity is linked to an abnormal expression of inhibitory killer immunoglobulin-like receptors (KIRs) [8].

From these observations, we conclude that available data do not allow us to predict NK phenotype in PV, although the development of an immune response against PV clone could be of great interest in the attempt to eradicate the disease. In order to answer this question we analyzed NK from PV patients in comparison with healthy donors (HD) regarding the expression of activating and inhibitory molecules, cytotoxic functions and gene expression patterns.

2. Materials and methods

2.1. Cell preparation and cultures

Peripheral blood mononucleated cells (PBMC) were isolated from blood samples using lymphocyte separation medium density gradient centrifugation (Eurobio Technologies, France). Purified NK cells were obtained after depletion using EasySep kit (StemCell Technologies, Vancouver, Canada). Purity of the NK cell fraction ($CD3^-/CD16^+/CD56^+$) obtained from PV patients was assessed by flow cytometry and varied from 64% to 86%. NK were expanded for three weeks from total PBMC cultured at $0.75 \cdot 10^6$ cells/ml with feeder EBV cells at $0.75 \cdot 10^6$ cells/ml in RPMI 1640 medium GlutaMAX with 10% fetal calf serum (FCS) in the presence of recombinant human IL-2 (1000 UI/mL) (Sigma, St Louis, MO). The K562 cell line was grown in RPMI 1640 medium with 10% FCS.

2.2. CD107a mobilization assay

NK cells were stimulated with K562 cells at ratio 1/1. The negative control was obtained with NK cells and medium. The positive control was obtained with NK cells stimulated with phorbol-12-myristate-13-acetate (PMA) (2.5 μg/mL) and ionomycin (0.5 μg/mL). The CD107a-FITC antibody was added directly to the wells at a concentration of 50 μL/mL and incubated overnight. After incubation, cells were labeled with $CD56^{PC5}$ for 15 min at 4 °C in the dark.

2.3. Immunophenotyping

Fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanin (PC5) coupled monoclonal antibodies (mAbs) were used to define the NK cells phenotype: CD3 (IgG1), CD16 (IgG1), CD56 (IgG1); activatory receptors and coreceptors: NCR3/NKp30 (IgG1), NCR2/NKp44 (IgG1), NCR1/NKp46 (IgG1), NKG2D (IgG1), CD244/2B4 (IgG1); inhibitory receptors: CD158a,h (IgG1), CD158b1,b2j (IgG1), CD158e1/e2 (IgG1), CD158i (IgG2a), CD85j/ILT2 (IgG1), CD159a/NKG2A (IgG2b). PBMC were stained with anti-CD3, anti-CD56, anti-CD158a,h (clone EB6) and anti-CD158a (clone 143211, IgG1, R&D Systems)-conjugated mAb. All antibodies were purchased from Beckman Coulter (Marseille, France). Total blood was incubated for 15 min at 4 °C, washed with PBS and analyzed on a XL-Epics (Beckman Coulter, Marseille, France). Background levels were measured using isotypic controls.

2.4. Quantification of intracellular perforin and granzyme

NK cells ($5 \cdot 10^5$) were first incubated during 15 min at 4 °C with 10 μL anti-$CD3^{FITC}$/$CD56^{PC5}$ and then fixed and permeabilized with Intraprep reagent (Beckman Coulter, Marseille, France). NK cells were incubated with anti-$perforin^{PE}$ (eBiosciences, San Diego, USA) or anti-granzyme B^{PE} (R&D Systems) during 15 min at room temperature.

2.5. Transcriptome analysis

Sample amplification, labeling, and hybridization followed the Low Input Quick Amp Labeling, one-color protocol (version 6.5, May 2010) recommended by Agilent Technologies. 500 ng of each total RNA sample was reverse transcribed into cDNA using oligo dT-T7 promoter primer. Labeled cRNA was synthesized from the cDNA. The reaction was performed in a solution containing dNTP mix, cyanine 3-dCTP, and T7 RNA Polymerase, and incubated at 40 °C for 2 h. Hybridization was performed into whole human genome microarray slides (4 × 44 K G4112F, Agilent Technologies, Santa Clara, CA) containing 45,220 oligonucleotide probes at 65 °C for 17 h. Hybridized microarray slides were then washed according to the manufacturer's instructions and scanned using an Agilent DNA Microarray Scanner, using the Agilent Feature Extraction Software (Agilent Technologies). Quantification files derived from the Agilent Feature Extraction Software were analyzed using the AgiND package (http://tagc.univmrs.fr/AgiND). We also used the AgiND R package for quality control and normalization. Quantile methods and a background correction were used for data normalization.

3. Results

3.1. Characteristics of patients and controls

After informed consent, blood samples were obtained from 17 patients with PV and from 15 aged-matched HD (66.3 years ±12.3 for PV patients vs 64.4 ± 9.3 for HD). The patients were diagnosed according to the world health organization (WHO) criteria. Median haemoglobin, platelets and leucocytes counts at the time of blood sample collection were 18.7 g/dL (range: 16.0–22.6 g/dL), 438 G/L (range: 210–643 G/L) and 12.1 G/L (range: 3.3–26.4 G/L), respectively. The *JAK2* V617F mutation was found in granulocytes of all patients and in 16 highly purified NK cells sample from patients and one patient had a mutation in exon 12 (F537-K539delinsL) (data not shown). PV patients were naive from any cytotoxic treatment at time of blood sampling.

3.2. Proportion and proliferation of NK cells in PV patients

Distribution of lymphoid populations in EDTA blood was compared between seventeen PV patients and fifteen HD and did not