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Cell surface cathepsin G activity differs between human natural killer cell subsets

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ABSTRACT

Natural killer (NK) cells are critical in diverse defense mechanisms, including elimination of viral infected cells and destruction of tumor cells. NK cells are characterized by the ability to initiate apoptosis in target cells when their cell surface major histocompatibility complex class I (MHC I) repertoire is missing. On the other hand, NK cells are not activated when MHC I or non-classical MHC molecules are found on the respective cells. It was demonstrated that cathepsin G (CatG) binds to the cell surface of NK cells; however, the distribution of this protease on the cell surface of NK cell subsets has not been identified. Here, we show that CatG cell surface level differs between NK cell subsets. CatG was determined on the protein- and activity level (activity-based probe MARS116) by using flow cytometry. Thus, MARS116 is a novel reporter of cell surface CatG activity and can be used to differentiate between distinct NK cell subsets.

1. Introduction

Natural killer (NK) cells [1], which comprise up to 15% of blood lymphocytes, control viral infected and tumor cells [2–4]. The aberrant cell surface expression of major histocompatibility complex class I (MHC I), mainly found in tumor or virus-infected cells, recognized by the killer-cell Ig-like receptor (KIR) provokes NK cell activation and secretion of cytokines/chemokines, granzymes, and perforins. Perforins penetrate the plasma membrane by forming a pore where granzymes can pass and induce apoptosis in the target cell. On the other hand, these cells are not activated when MHC I- or non-classical MHC molecules are recognized on the cell surface [5–7]. NK cells are characterized by the absence of the T lymphocyte marker CD3 and are divided to several subpopulations depending on their CD16 and CD56 cell surface expression [8,9]. While $CD16^{+}CD56^{dim}$ have cytotoxic capacity, $CD16^{+}CD56^{bright}$ NK cells have a regulatory function which is comparable to T regulatory cells [10,11].

Activated polymorphonuclear neutrophils secret serine proteases: cathepsin G (CatG), neutrophil elastase (NE), and proteinase 3 (PR3) during an immune response [12]. CatG is not only involved in physiological processes, under certain circumstances CatG is responsible for several pathophysiological cases, including autoimmunity and cancer [13,14]. Interestingly, CatG is found on the cell surface of different immune cells, more precisely, T cells, B cells, NK cells, and activated pro-inflammatory monocytes [15,16]. However, it is not known which NK cell subsets bind CatG and whether cell surface CatG of these cells is also proteolytic active.

The active-site label is based on the detection of protease activity by an inhibitor which binds covalently to the active center of the respective protease. The incorporation of biotin or a fluorophore to the inhibitor (called activity-based probe) allows the visualization of a catalytically active protease [17,18]. MARS116 contains a phosphonate as the reactive functional group and is suitable for visualizing CatG activity. Thereby, the serine residue of the catalytic center of CatG performs a nucleophilic attack to the electrophilic phosphorus atom and CatG can be resolved in a Western blot-based approach (active-site label) [19]. Furthermore, activity-based probes can be used in flow cytometry analysis as demonstrated for cysteine-aspartic proteases (caspases) where a caspase inhibitor was labeled with fluorescein isothiocyanate (FITC) and H_2O_2-induced apoptosis was analyzed in Jurkat cells by flow cytometry [20]. However, one cavity of such an activity-

Abbreviations: Caspase, cysteine-aspartic protease; Cat, cathepsin; KIR, killer-cell Ig-like receptor; MARS116, **Mar**cin **S**ienczyk 116, Bt-LC-Suc-Val-Pro-PheP(OPh)2; MFI, median fluorescence intensity; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; NE, neutrophil elastase; NK cells, natural killer cells; PBMCs, peripheral blood mononuclear cells; PR3, proteinase 3, SDF-1, chemokine stromal cell-derived factor 1.

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based probe is selectivity; therefore, Verdoes et al. generated a non-peptide CatS activity-based probe which selectively visualizes the cysteine protease CatS in macrophages [21]. Certainly, an activity-based probe to detect also serine proteases, such as CatG, in flow cytometry is needed. Here, we demonstrate that the application of MARS116 is a novel strategy to determine CatG activity at the cell surface. Moreover, these data suggest that the proteolytic activity of CatG might be important for $CD16^{-}CD56^{dim}$ and $CD16^{dim}CD56^{-}$ NK cell function.

2. Materials and Methods

2.1. Assessment of CatG at the cell surface of NK cells

Freshly purified (n = 10) or cryopreserved (n = 39) PBMCs from healthy donors (young, 18-25 years and elderly, 59-70 years) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were washed with PBS pH 7.4 and adjusted to a final concentration of 5×10^6 PBMCs/ml. Subsequently, anti-CD3-PerCP, anti-CD56-PE (BD Biosciences, Minneapolis, MN, USA), anti-CD16-APC, (eBioscience, San Diego, California, USA), and anti-CatG-FITC (Acris Antibodies GmbH, Herford, Germany) were diluted in blocking buffer (1% FBS in PBS), and PBMCs were stained for 30 min at 4 °C. After two wash steps, cells were collected by a BD Canto II cytometer (Franklin Lakes, NJ, USA) and cells were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA). Statistical analysis was done by GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). Data were depicted as S.D. and statistical analysis was performed using one-way analysis of variance (ANOVA). Additionally, multiple comparisons were adjusted by the Bonferroni correction (Prism 6, GraphPad Software, La Jolla, CA, USA). Use of PBMCs for *in vitro* studies has been approved (ethics committee, Ulm University, Helmholtzstr. 20, 89069 Ulm, Germany, proposal # 327/14).

2.2. Application of MARS116 to determine CatG activity at the cell surface of NK cells

5×10^6 PBMCs/ml were incubated with 2 μM of the activity-based probe Bt-LC-Suc-Val-Pro-$Phe^{P}(OPh)_2$ (Marcin Sienczyk 116, MARS116 [19]) or with different concentrations of the CatG inhibitor 1 (Calbiochem, Schwalbach, Germany, [22]) in PBS pH 7.4 for 30 min at room temperature (RT). Cells were washed twice with PBS (pH 7.4) and incubated with 50 μg/ml avidin D-FITC (Vector Laboratories, Burlingame, CA, USA) for 15 min at RT. Afterwards, cells were prepared for flow cytometry as described before. In a control experiment, PBMCs (5×10^6 PBMCs/ml) were incubated with different concentrations of CatG from human neutrophils (BioCentrum Ltd., Krakow, Poland) and CatG activity was determined as described above.

3. Results

3.1. CatG is expressed on the cell surface of $CD16^{-}CD56^{dim}$, $CD16^{bright}CD56^{-}$, and $CD16^{dim}CD56^{-}$ cells

It was demonstrated that CatG can bind to the cell surface of NK cells [15,16]. Having this in mind, we addressed the question whether NK cell subsets differ in their cell surface CatG repertoire. To this end, human peripheral blood mononuclear cells (PBMCs) were incubated with a specific CatG antibody conjugated with FITC and levels of CatG were analyzed by flow cytometry. NK cells were similarly gated as previously suggested [23]: Cells were gated for $CD3^{-}$ and followed by $CD16^{-}CD56^{bright}$ (1), $CD16^{dim}CD56^{bright}$ (2), $CD16^{-}CD56^{dim}$ (3), $CD16^{bright}CD56^{dim}$ (4.1), $CD16^{dim}CD56^{dim}$ (4.2.), $CD16^{bright}CD56^{-}$ (5.1), or $CD16^{dim}CD56^{-}$ (5.2) (Fig. 1A). Strikingly, $CD16^{-}CD56^{dim}$ (3), $CD16^{bright}CD56^{-}$ (5.1), and $CD16^{dim}CD56^{-}$ (5.2) NK cells showed high levels of CatG in contrast to $CD16^{-}CD56^{bright}$ (1), $CD16^{dim}CD56^{bright}$ (2), $CD16^{bright}CD56^{dim}$ (4.1), and $CD16^{dim}CD56^{dim}$ (4.2.) (Fig. 1 B and 1C). Moreover, we distinguished CatG on NK cell subsets from young and elderly donors and found that CatG is slightly elevated on $CD16^{dim}CD56^{-}$ (5.2) from young female donors (Supp. data 1). Thus, CatG is present on the cell surface of $CD16^{-}CD56^{dim}$ (3), $CD16^{bright}CD56^{-}$ (5.1), and $CD16^{dim}CD56^{-}$ (5.2) NK cells.

3.2. Assessment of CatG activity at the cell surface of NK cells

To investigate whether CatG bound to the cell surface of $CD16^{-}CD56^{dim}$ (3), $CD16^{bright}CD56^{-}$ (5.1), and $CD16^{dim}CD56^{-}$ (5.2) NK cells is proteolytic active, cell surface active-site label for flow cytometry was established. PBMCs were incubated with the biotinylated activity-based probe MARS116 and stained with the respective NK cell markers. CatG activity was visualized by using avidin D-FITC. However, specific CatG activity was only statistically significant for $CD16^{-}CD56^{dim}$ (3) and $CD16^{dim}CD56^{-}$ (5.2) NK cells as determined by using the CatG inhibitor (Fig. 2). These data indicate that levels of CatG do not reflect the proteolytic activity of CatG of the $CD16^{bright}CD56^{-}$ (5.1) NK cell subset. In order to determine the specificity of the cell surface labeled CatG, PBMCs were incubated with MARS116, cells were harvested, and CatG activity was detected in a Western blot-based active-site label (Supp. Data S2). Furthermore, different concentrations of exogenous CatG were added to PBMCs. CatG activity was detected with MARS116 when PBMCs were preincubated with purified CatG in a concentration dependent manner (Fig. 3). Taken together, CatG detected on $CD16^{-}CD56^{dim}$ (3) and $CD16^{dim}CD56^{-}$ (5.2) NK cells is also proteolytic active.

4. Discussion

NK cells, belonging to the cytotoxic type 1 innate lymphoid cells, are important for an anti-tumor immune response [11]. Indeed, it was demonstrated by screening 3500 healthy individuals that tumor growth decreases with the cytotoxicity of NK cells [24]. As they develop, NK cells undergo, most likely, differentiation from naïve $CD56^{bright}$ to mature $CD56^{dim}$ NK cells [25,26]. Among these NK cell subsets, $CD56^{bright}$ NK cells efficiently secrete cytokines and chemokines with immunoregulatory capacity, whereas $CD56^{dim}$ NK cells are known for their effective cytotoxicity [10,11]. Functional differences have been found between the $CD16^{bright}$ NK subpopulation and $CD16^{dim}$ or $CD16^{-}$ NK cells. While the $CD16^{bright}$ NK subset shows a highly antibody-dependent cytotoxic effector function, the $CD16^{dim}$ or $CD16^{-}$ subsets are less or not antibody-dependent cytotoxic, respectively [8,23]. Although NK cells did not express CatG on the transcriptional level, CatG activity was visualized by active-site label (Supp. Data S3). We found that CatG is proteolytic active at the cell surface of $CD16^{-}CD56^{dim}$ (3) and $CD16^{dim}CD56^{-}$ (5.2) indicating that MARS116 can be used as an additional marker to determine NK cell subsets. CatG on these cells might cause harm since active cell surface CatG is responsible for the conversion of pro-matrix metalloproteinase 9 (MMP-9) to active MMP-9, and MMP-9 itself activates TGF-β [27,28], which leads to the assumption that these NK cell subsets might be under certain circumstances pro-tumorigenic. However, it was shown that cell surface bound CatG deactivates surface chemokine stromal cell-derived factor 1 (SDF-1) by proteolysis [16]. In general, high levels of SDF-1 were found in glioblastoma [29], which supports the statement for a tumor suppressive role of the proteolytic activity of cell surface bound CatG.