Journal of Autoimmunity xxx (2016) 1–11

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Dysregulation of regulatory CD56^{bright} NK cells/T cells interactions in multiple sclerosis

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ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form
15 April 2016
Accepted 19 April 2016
Available online xxx

Keywords:
Multiple sclerosis
NK cells
CD56^{bright} NK cells
Innate immune cells

ABSTRACT

Recent evidence has shown that CD56^{bright} NK cells, a subset of NK cells abundant in lymph nodes, may have an immunoregulatory function. In multiple sclerosis (MS), expansion of CD56^{bright} NK cells has been associated to successful response to different treatments and to remission of disease during pregnancy; how whether they exert immunoregulation in physiologic conditions and whether this is impaired in MS is not known. We dissected the immunoregulatory role of CD56^{bright} NK cells function in healthy subjects (HS) and compared it with that of untreated MS subjects or patients with clinically isolated syndrome suggestive of MS (CIS). We found that CD56^{bright} NK cells from HS acquire, upon inflammatory cues, the capability of suppressing autologous CD4+T cell proliferation through direct cytotoxicity requiring engagement of natural cytotoxicity receptors (NCRs) and secretion of granzyme B. CD56^{bright} NK cells from patients with MS/CIS did not differ in frequency and share a similar phenotype but displayed a significantly lower ability to inhibit autologous T cell proliferation. This impairment was not related to deficient expression of NCRs or granzyme B by CD56^{bright} NK cells, but to increased HLA-E expression on T cells from MS/CIS subjects, which could enhance the inhibitory effect mediated by NKG2A that is homogeneously expressed on CD56^{bright} NK cells. The defect in controlling autologous T cells by CD56^{bright} NK cells in MS/CIS might contribute to the excess of autoimmune response that is associated to disease development.

1. Introduction

In recent years, the horizon of innate immune cells has been redesigned with the identification of innate lymphoid cells (ILC). ILC are now regarded as the innate, more phylogenetically ancient mirror of T cells and thus, based on transcription factors and cytokines they produce, ILC are divided in subfamilies similar to T helper cell subsets [1]. However, whether ILC include the innate correlate of T regulatory cell subsets has not been clarified yet.

Natural killer (NK) cells are type 1 ILC innate cells with prominent antitumoral and antiviral functions, which they exert through direct cytotoxicity. NK cells express activating receptors, which bind non HLA-specific ligands [2,3] and inhibitory receptors [4,5] which mainly bind to autologous HLA class I molecules, thus preventing them to attack healthy, autologous cells. Human NK cells can be divided into functionally distinct subsets, based on the levels

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http://dx.doi.org/10.1016/j.jaut.2016.04.003

of CD56 and of many other receptors crucial to carry out their effector functions. Most circulating NK cells are $CD56^{dim}$ $CD16^{+}$, KIR+ and/or NKG2A+ and can kill target cells and produce cytokines following specific recognition of their targets [6–8]. By contrast, $CD56^{bright}$ NK cells, which are the predominant NK cell subset in peripheral lymphoid organs, are CD16-/low, KIR-, NKG2A+, and are known to produce large amounts of cytokines but to acquire cytotoxicity only after prolonged activation [6,7].

We previously showed that IL-27-stimulated $CD56^{bright}$ NK cells suppress autologous $CD4^{+}$ T cell proliferation [9] and, more recently, Morandi and coauthors showed that $CD56^{bright}$ NK cells, in presence of autologous $CD4^{+}$ T cells, release adenosine that decreases T cell proliferation [10]. However, the triggers and mechanisms of such immunosuppressive function, and whether $CD56^{bright}$ NK cells may be the counterpart of T regulatory cells within the ILC compartment, are still to be elucidated.

Defects in regulatory cell populations of the adaptive immune system, and particularly regulatory T cells (Tregs) have been linked to the generation of autoimmune responses. In particular, impairment in Tregs, such as natural Tregs, induced Tregs or T regulatory cells type 1 (Tr1 cells) may link to the deregulated immune activation which leads to multiple sclerosis (MS), an autoimmune disease of the central nervous system (CNS) [11]. Recent clinical evidence suggests that $CD56^{bright}$ NK cells might also be able to counteract autoimmune responses in the CNS. In this context, higher numbers of $CD56^{bright}$ NK cells have been observed in subjects with MS treated with interferon-beta, as well as MS patients during pregnancy, a condition associated to remission of disease activity [12,13]. More importantly, the efficacy of the anti-CD25 monoclonal antibody, daclizumab, in MS was directly linked to an increase in the number of circulating $CD56^{bright}$ NK cells [14]. In patients treated with daclizumab, there was a direct correlation between an increase of $CD56^{bright}$ NK cells and reduction in the number of active lesions at Magnetic Resonance Imaging (MRI). This was linked to an increased availability of IL-2, not binding to T cells, that could activate $CD56^{bright}$ NK cells through the intermediate-affinity receptor [15]. $CD56^{bright}$ NK cells from daclizumab-treated patients acquired a cytotoxic effect towards autologous T cells that was mediated by the lytic enzyme granzyme K [14,16].

However, it is not known whether $CD56^{bright}$ NK cells exert relevant regulatory functions in physiologic conditions, to what extent such function is important to prevent excess of immune activation, and whether the function of $CD56^{bright}$ NK cells is impaired in MS. Accordingly, the aim of this study was to assess the possible regulatory function of $CD56^{bright}$ NK cells in healthy people and in patients with MS/CIS.

2. Material and methods

2.1. Study design and population

We based our estimate of the sample size required for the study from our previous study with IL-27 stimulated $CD56^{bright}$ NK cells, where we observed a mean suppression of T cell proliferation = 46% with a SD = 2. To observe a similar suppressor function of $CD56^{bright}$ NK cells after IL-12 and IL-15, in HS, with a power = 0.90 and a significance level = 0.05, we needed a sample size of N = 4. To detect a 30% reduction in the suppressor function of $CD56^{bright}$ NK cells from CIS/MS compared to HS, we needed a sample size N = 8. Therefore, we planned to enroll 10 HS and 10 MS or CIS patients.

PBMCs were isolated through Ficoll Paque centrifugation. In some cases, PBMCs were frozen in Fetal Calf Serum (FCS) 90% plus dimethyl sulfoxide (DMSO) 10%. We enrolled a total number of 22 patients with relapsing-remitting MS or CIS (MS/CIS patients) in the study. F/M ratio was 14/8, mean age at enrolment (standard deviation–SD) was 33.04 (9.5) years, mean time after first symptom suggestive of MS (SD) was 4.9 (7.2) years (not available for one patient). None of the patients was under treatment with disease modifying drugs nor steroids at the time of sampling and all had been untreated for at least one month before blood sampling. Twenty-one healthy donors, female/male ratio 11/10, mean age = 35.00 years (9.9), donated peripheral blood. In addition, buffy coats/leukopaks (HS) from anonymous blood donors were employed. The amount of peripheral blood drawn from patients or HS for the study was 70 ml.

Experiments were carried out in two laboratories. For experiments carried out at the Brigham and Women's Hospital, peripheral blood leukopak cells were obtained from Children's Hospital, Boston MA. The leukopak cells are obtained at the time of routine blood donation in which subjects provide written consent to have blood drawn. These procedures are in accordance with the Children's Hospital Institutional Review Board. Peripheral blood leukopak cells were analyzed in the laboratory at the Brigham and Women's Hospital that is approved by the institutional review board at Brigham and Women's hospital for the study of human blood. For experiments carried out at the University of Genova, peripheral blood (HS, MS/CIS patients) was obtained after patients had provided their informed consent. The study was approved by the Ethical Committee of IRCCS Azienda Ospedaliera Universitaria San Martino-IST (Protocols N. 106/11 and 190/12). Buffy coats (HS) from anonymous blood donors were obtained from the blood bank of IRCCS San Martino IST.

2.2. Cell sorting and flow cytometry

For cell sorting and flow cytometry, the following commercially, anti-human antibodies were used: CD56 (Biolegend HCD56 PE/Cy7, APC), CD56 (Biolegend MEM-188 PE/Cy7) (Beckman Coulter IgG1 PC7), CD3 (Biolegend UCHT1, Pacific Blue, FITC), CD16 (BD 3G8 IgG1 Per CP-Cy 5.5, APC), CD25 (BD IgG1 Brillant Violet 421), CD25 (Biolegend BC96 APC/Cy7), CD107a (Biolegend, H4A3 IgG1 PE), CD69 (Biolegend FN30 PE), Granzyme B (BD Pharmigen GB11 IgG1 FITC), anti-CD4 (Biolegend clone SK3 PerCP), anti-HLA-E (Biolegend clone 3D12 IgG1 primary antibody).

Primary anti-NKG2A (Z199 IgG2b), anti-KIRs (AZ158, IgG2a; XA141 and Y249, IgM), anti-NCRs (aNKp30: AZ20, IgG1; aNKp44: Z231, IgG1; aNKp46: BAB281, IgG1) mAbs, produced in our laboratory (Department of Experimental Medicine, University of Genova), were used in combination with secondary anti-mouse IgG1, IgM, IgG2a PE, IgG2b FITC (Southern Biotechnology) and IgG2a APC (Jackson Immunoresearch) conjugated antibodies. All the primary antibodies used were produced in AM laboratory. Cell viability was evaluated with 7-AAD (Cell Viability Solution BD Biosciences). For quantification of the number of antibodies bound per cell (ABC), cells were stained with the primary antibody anti-NCRs or anti-HLA E and secondary conjugated PE antibody. Then PE fluorescence quantification kit (QuantiBRITE, Becton Dickinson) was used for a flow cytometric estimation for antibodies bound per cell. Briefly, when a QuantiBRITE PE tube is run at the same instrument settings as the assay, the mean fluorescent intensity of the FL2 axis can be converted into the number of PE molecules bound per cell. With this method it is possible to create a standard curve to calculate the number of antibodies bound per cell (ABC) and consequently, the receptor number present on the cell surface.

In order to assess the intracellular content of granzyme B, $CD4^{+}$ cells and pre-activated $CD56^{bright}$ NK cells, alone or together, were cultured overnight in presence of anti-CD3/anti-CD28 beads (Life Technologies). Cells were then washed, stained for surface expression of CD4 and CD56, fixed and permeabilized (BD cytofix/

Please cite this article in press as: A. Laroni, et al., Dysregulation of regulatory $CD56^{bright}$ NK cells/T cells interactions in multiple sclerosis, Journal of Autoimmunity (2016), http://dx.doi.org/10.1016/j.jaut.2016.04.003