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

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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 of CD56 and of many other receptors crucial to carry out their effector functions. Most circulating NK cells are CD56^{dim} CD16^{+},

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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/cytoperm kit) and fluorescent anti-human granzyme B antibody was added [16]. Intracellular content of granzyme B in CD4$^+$ T cells

alone vs $CD4^+$ T cells cultured in presence of $CD56^{bright}$ NK cells, and in $CD56^{bright}$ NK cells alone vs $CD56^{bright}$ NK cells cultured with $CD4^+$ T cells was analyzed with the Student's *t*-test.

Cells were sorted with a BD FACS Aria cell sorter. Flow cytometry data were acquired through a BD FACS Canto II. Analysis of flow cytometry data was performed with Flow Jo version 8 for Macintosh and version 7 for Windows.

2.3. Isolation of NK cells

NK cells were negatively isolated from fresh or frozen PBMCs (Miltenyi human NK cells isolation kit); NK cells were divided into $7\text{-AAD}^{negative/}CD3\text{-}CD56^{bright}$ and $7\text{-AAD}^{negative}CD3\text{-}CD56^{dim}$ NK cells by cell sorting. We chose to sort NK cell subsets based on the expression of CD56 only in order to avoid triggering or masking receptors involved in NK cell functions. Purity of NK cells was assessed by flow cytometry after cell sorting and was equal to 99%.

2.4. Isolation of $CD4^+$ T cells

$CD4^+$ T cells were negatively isolated from fresh or frozen PBMCs (Miltenyi human $CD4^+$ T cell isolation kit). Purity of $CD4^+$ T cells after isolation was checked by flow cytometry to be above 95%. $CD4^+$ T cells were frozen in fetal calf serum (FCS) 90% plus dimethyl sulfoxide (DMSO) 10%, during the 3-days activation period of NK cell subsets detailed below.

2.5. Activation of NK cells and suppression assay

NK cell subsets were cultured in fresh medium alone or with human recombinant IL-12 (R and D Systems, 10 ng/ml) plus human recombinant IL-15 (R and D systems, 100 ng/ml) for 72 h. To study gene expression, the culture was stopped after 24 h. Activated living (7-AAD negative) NK cells were resorted and cultured for 5 days in presence of sorted living (7-AAD negative) autologous $CD4^+$ T cells at a 1:1 ratio in presence of anti-CD3 anti-CD28 activation beads (Life Technologies); controls included activating beads plus CD4 + T cells alone or activating beads plus NK cells alone. Alternatively, living NK and $CD4^+$ T cells were counted through optical microscopy in presence of Trypan blue and cocultured as previously detailed. To suppress the effect of granzyme B, the specific inhibitor Z-AAD-CMK (Merck Millipore) was added to the cultures at concentration = 10 nM.

Proliferation of T cells was assessed by incorporation of ^3H thymidine or with the CyQUANT Cell Proliferation Assay Kit, that measures the DNA content in cultures (Life Technologies), subtracting the measure obtained from $CD56^{bright}$ NK cells cultured in absence of T cells. Proliferation of $CD56^{bright}$ NK cells was not observed in control wells when measured by incorporation of ^3H thymidine.

In order to compare the suppressor function of $CD56^{bright}$ NK cells from HS and MS/CIS patients, paired experiments (one or two MS/CIS subjects, one or two HS) were carried out.

2.6. Degranulation assay

For cytotoxicity assay, NK cell subsets and autologous $CD4^+$ T cells were cocultured in presence of anti-CD3 anti-CD28 activation beads, as described above. Cultures were stopped after 24 h to assess degranulation of NK cells and after 5 days to assess death of CD4+T cells. For degranulation assays, anti-CD107a antibody and brefeldin A (Sigma) were added to the wells, respectively 4 and 3 h before stopping the culture. Cells were then washed and stained with fluorescent antibodies (anti-CD3, anti-CD56, and anti-CD16) for surface markers. Expression of CD107a on NK cells cultured alone was used as negative control. For analysis of T cell death, the % of 7-AAD-positive, CD3-positive cells was assessed.

2.7. Study on gene expression

Total RNA was isolated from cell pellets using RNAeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed for each RNA sample from 0.5 to 1 μg of total RNA using Taqman reverse-transcription reagents (Life Technologies). For experiments carried out at the Brigham and Women's hospital laboratory, cDNA was amplified using sequence-specific primers and real-time PCR mix (Applied Biosystems) on ABI 7500 cycler. For experiments carried out at University of Genova, real-time PCR was performed using a LightCycler 480 (Roche) in presence of SYBR Green I Master mix (Roche), according to the manufacturer's instruction and of the following primers, synthesized by Tib Molbiol (Genova, Italy), were used: human granzyme A: forward primer 5′-TTAACCCTGT-GATTGGAATGAAT-3′, reverse primer 5′-AGGGCTTCCAGAATCTC-CAT-3′; human granzyme B, forward primer 5′-AGATGCAACCAATCCTGCTT-3′, reverse primer 5′-CATGTCCCCC-GATGATCT -3′ human granzyme H forward primer 5′-GCAGCCTTCCTGAGAAAATG-3′, reverse primer 5′-CCCCGAT-GATCTCCTCTGT-3′; human granzyme K: Forward primer 5′-ATCTGGAACCAAATGCAAGG-3′, Reverse primer 5′-TCTCGCAGGGTGTCAGAAG; human granzyme M forward primer 5′-GCGTGTCTTCACTGCTGGT-3′, reverse primer 5′-ATGATCTGGGTCC-CAAAGg-3′; human perforin forward primer 5′-CCGCTTCTCTA-TACGGGATTC, -3′ reverse primer GCAGCAGCAGGAGAAGGA-3′; human Fas ligand: forward primer 5′-TGGGGATGTTTCAGCTCTTC-3′, reverse primer 5′-TGTGCATCTGGCTGGTAGAC-3′; human CD16 forward primer 5′-GGGGGCTTTTTGGGAGTA-3′, reverse primer 5′-GGTTGACACTGCCAAACCTT-3′.

The human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer 5-AGCCACATCGCTCAGACAC-3′, reverse primer 5′-GCCCAATACGACCAAATCC-3′) was used as housekeeping gene to normalize the expression data. All values were expressed as relative expression of gene of interest to the expression of GAPDH and displayed as ratio of stimulated vs. unstimulated cells.

2.8. Receptor blocking experiments

To evaluate the possible contribution of NK cell receptors to suppression of proliferation of autologous T cells, suppression assays were carried out in presence or absence of the following antibodies produced in our laboratory (Department of Experimental Medicine, University of Genova): anti-NKp30 (F252, IgM), anti NKp44 (KS38, IgM), anti-NKp46 (KL247, IgM), anti DNAM-1 (F5, IgM) anti NKG2D, (BAT221 IgG1), anti HLA-I (A6136, IgM). The anti-HLA I antibody was allowed to bind to $CD4^+$ T cells before adding $CD56^{bright}$ NK cells, while anti-NKp30, anti-NKp44, anti-NKp46, anti-NKG2D and anti-DNAM-1 were added to the co-cultures since the beginning. To study the effects of blocking HLA-class I in $CD4^+$ T cells from HS compared to MS/CIS patients, paired experiments (one or two MS/CIS patients, one or two HS) were carried out. F252, F5 and BAT221 mAbs were kindly provided by Dr. D. Pende from IRCCS San Martino-Ist, Genova, Italy. KL247 and KS38 mAbs were kindly provided by Prof. S. Parolini from University of Brescia, Italy.

2.9. Statistics

Sample size was calculated with the PASS 11 software. Data were analyzed with Graph Pad Prism 6. The following statistic tests were used: two tailed Wilcoxon matched-pairs test, Student *t*-test, Mann-Whitney test. A $P \leq 0.05$ was considered significant.

3. Results

3.1. IL-12 and IL-15 induce immunoregulatory cytotoxic CD56^{bright} NK cells

NK cells were negatively isolated from fresh or frozen peripheral blood mononuclear cells (PBMCs) and NK cell subsets were sorted by the expression of CD56. The phenotype of CD56^{bright} (Supplementary Fig. 1) and CD56^{dim} (not shown) NK cell subsets was assessed by flow cytometry after cell sorting.

We chose to activate CD56^{bright} NK cells with IL-12 and IL-15, two antigen presenting cells (APC)-derived, proinflammatory cytokines whose levels are increased in MS and having a pivotal role in NK activation [17–19]. After activation with IL-12 and IL-15, the two CD56^{bright} and CD56^{dim} NK cell subsets were cocultured for 5 days with autologous CD4$^+$ T cells in presence of a T cell specific stimulus, represented by anti-CD3/anti-CD28 beads. We observed that CD56^{bright}, but not CD56^{dim} NK cells significantly suppressed autologous CD4$^+$ T cells proliferation ($P = 0.002$) (Fig. 1a). We did not observe significant suppression of proliferation of autologous T cells when co-culturing them with unstimulated CD56^{bright} NK cells (Supplementary Fig. 2).

We investigated whether the decreased proliferation of CD4$^+$ T cells in presence of CD56^{bright} NK cells was due to increased cell death. We measured by flow cytometry the percentage of dead (7-aminoactinomycin D+)(7-AAD+) T cells in presence of activated CD56^{bright} and CD56^{dim} NK cells after 5 days of coculture with anti-CD3/anti-CD28 beads and observed a significantly higher % death of CD4$^+$ T cells in presence of CD56^{bright} NK cells ($P = 0.03$), but not CD56^{dim} NK cells ($P =$ ns) (Fig. 1b).

We postulated that CD56^{bright} NK cells-mediated cytotoxicity could be responsible for increased death of autologous T cells. Therefore, we studied degranulation of CD56^{bright} NK cells (as measured by expression of CD107a on NK cell surface) after one day of coculture with autologous CD4$^+$ T cells and anti-CD3/anti-CD28 beads, in comparison with that of CD56^{dim} NK cells. We observed a significantly higher degranulation of CD56^{bright} NK cells (Fig. 1c, central panel and Fig. 1d) compared to CD56^{dim} NK cells (Fig. 1c, left panel and Fig. 1d). CD56^{bright} NK cells did not degranulate in presence of unstimulated CD4$^+$ T cells, thus indicating that this NK-mediated effector function was specific for CD4$^+$ T cells undergoing activation (Fig. 1c, right panel and 1e). To assess whether a cell-to-cell contact cytotoxic mechanism was involved in the suppressive function of CD56^{bright} NK cells, we assessed suppression in contact or transwell experimental condition, and found that transwell coculture abrogated the suppression of autologous CD4$^+$ T cell proliferation by CD56^{bright} NK cells (Fig. 1f). These results suggest that a direct cell-to-cell interaction is required to allow the suppressive function of CD56^{bright} NK cells against autologous activated T cells.

3.2. Natural cytotoxicity receptors are required for CD56^{bright} suppressive function

In order to assess which receptors trigger the suppressive function of CD56^{bright} NK cells against autologous activated T cells, we performed proliferation assays in presence of antibodies masking the main non HLA-specific activating NK receptors (natural-killer group 2, member D- NKG2D, natural cytotoxicity receptors -NCRs: NKp30, NKp44, NKp46, DNAX Accessory Molecule-1- DNAM-1) or blocking HLA class I molecules in T cells. We chose not to block the activating CD16 receptor because in our in vitro culture system, a role of CD16 is unlikely, since the receptor is involved in antibody-dependent cell-mediated cytotoxicity and there were no antibodies added to the co-culture (except for those bound to the magnetic beads for stimulation of T cells: these beads-associated mAbs are unable to bind to CD16) [20]. We found that the suppressive function of CD56^{bright} NK cells was reverted by addition of a mix of anti-NCR antibodies ($P = 0.0005$), while blocking other activation receptors (including NKG2D and DNAM-1) did not restore T cell proliferation (Fig. 2a). Masking the HLA molecules on T cells did not increase significantly the cytotoxicity of NK cells towards T cells (Fig. 2a). To determine what NCRs were specifically involved in NK-T interaction, we performed suppression assays in presence of antibodies blocking single NCRs and observed that anti-NKp30 and anti-NKp46 antibodies, but not anti-NKp44 antibody, reverted CD56^{bright} suppressor function (Fig. 2b). In addition, we evaluated the number of NCRs expressed in basal conditions or upon exposure to IL-12 and IL-15 by CD56^{bright} NK cells and found that NKp30 and NKp44, but not NKp46, were upregulated after cytokines-mediated activation (Fig. 2c). We did not observe upregulation of the NKp46 receptor that had already high baseline expression in CD56^{bright} NK cells, consistently to what is known from the literature [21,22].

3.3. Release of granzyme B by CD56^{bright} NK cells is involved in cytotoxicity of autologous CD4$^+$ T cells

To dissect cytotoxicity of CD56^{bright} NK cells, we evaluated, by real-time PCR, changes in transcription of genes encoding granzyme proteins, perforin, Fas ligand, the Fc-gamma receptor CD16, and theTNF-related apoptosis-inducing ligand (TRAIL), all of which are involved in NK-mediated cytolytic activity. We observed that IL-12 and IL-15 upregulated exclusively mRNA for granzyme B in CD56^{bright} NK cells while other genes were not induced (Fig. 3a). Then, we evaluated the intracellular content of granzyme B in CD4$^+$ T cells and CD56^{bright} NK cells cultured separately or together, similarly to what previously described by Jiang and coauthors [16]. In CD4$^+$ T cells/CD56^{bright} NK cells cocultures, the intracellular content of granzyme B increased in CD4$^+$ T cells and decreased in CD56^{bright} NK cells, compared to CD4$^+$ T cells or CD56^{bright} NK cells cultured alone, thus suggesting a transfer of granzyme B from NK cells to T cells during coculture (Fig. 3b).

Accordingly, inhibition of granzyme B function by Z-Ala-Ala-Asp-chloromethylketone (Z-AAD-CMK) significantly decreased the suppressive function of CD56^{bright} NK cells (Fig. 3c). Taken together, these three observations suggest that transfer of granzyme B mediates the cytotoxic function of CD56^{bright} NK cells towards autologous activated CD4$^+$ T cells.

3.4. Suppression of autologous T cells by CD56^{bright} NK cells is impaired in untreated MS individuals

Next, we sought to address whether CD56^{bright} NK cells from MS subjects could suppress autologous CD4$^+$ T cell proliferation as efficiently as NK cells from HS.

We decided to focus our analysis on untreated MS/CIS patients, rather than on treated MS patients, in order to assess whether a defect in the regulatory function of CD56^{bright}, which might contribute to disease development, is found in MS patients compared to healthy control.

We first observed that untreated MS patients and subjects with clinically isolated syndrome suggestive of MS (CIS) display proportion of CD56^{bright}/CD56^{dim} NK cells similar to those of HS (Fig. 4a). Subsequently, we assessed the capability of cytokines stimulated CD56^{bright} NK cells from untreated MS/CIS patients to suppress autologous T cell proliferation. Interestingly, we observed that the suppression of T cell proliferation by CD56^{bright} NK cells from untreated MS/CIS patients was significantly lower than that by CD56^{bright} NK cells from HS (Fig. 4b).

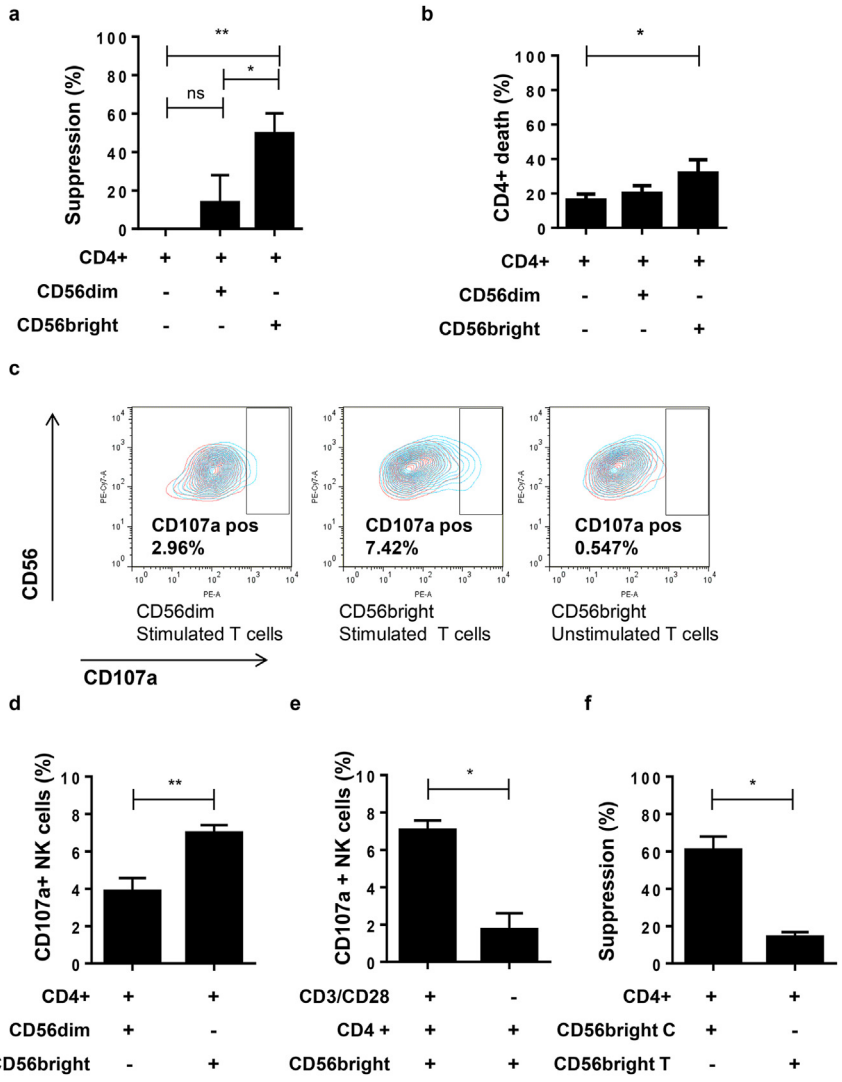

Fig. 1. CD56^{bright} NK cells suppress autologous CD4^{+} T cell proliferation through direct cytotoxicity. a. Plots showing suppression of autologous CD4^{+} T cell proliferation by CD56^{bright} NK cells (Wilcoxon, $P = 0.002$) but not CD56^{dim} NK cells (Wilcoxon, P= NS). There is a significant difference among the suppressive function of the two NK cell subsets (Wilcoxon, $P = 0.03$). Results are shown as mean ± standard error of the mean. N = 11 healthy subjects. b. Percentage of dead (7-AAD positive) CD4^{+} T cells in presence of CD56^{bright} NK cells (Wilcoxon, $P = 0.03$) and in presence of CD56^{dim} NK cells (Wilcoxon, P= NS) evaluated by flow cytometry. Results are shown as mean ± standard error of the mean. N = 6 experiments. c. Representative FACS plots showing degranulation of CD56^{dim} in presence of stimulated autologous CD4^{+} T cells (in blue, left), as compared with degranulation of CD56^{bright} NK cells (in blue, center). Right panel shows degranulation of CD56^{bright} NK cells in presence of non-activated autologous CD4^{+} T cells (in blue). Contour plots in red: same NK cell subset cultured in absence of autologous CD4^{+} T cells. d. Cumulative plots showing percentage of CD107a+ (degranulating) CD56^{bright} NK cells compared to percentage of CD107a + CD56^{dim} NK cells co-cultured with autologous CD4^{+} T cells (Wilcoxon test, $P = 0.01$). Results are shown as mean ± standard error of the mean. N = 9 healthy subjects. e. Plots showing percentage of degranulating CD56^{bright} NK cells in presence of activated (by CD3/CD28 molecules) and non activated CD4^{+} T cells Paired t-test, $P = 0.02$. Results are shown as mean ± standard error of the mean. N = 3 healthy subjects. f. Suppressive function of CD56^{bright} NK cells in presence of cell-to-cell contact (CD56^{bright} C) or in transwell (CD56^{bright} T) experimental condition. Results are shown as mean ± standard error of the mean. Paired t-test, $P = 0.04$. N = 3 healthy subjects.

3.5. CD56^{bright} NK cells from MS patients and from HS display a similar cell surface phenotype

Next, we sought to exclude that a defective suppressive function by CD56^{bright} NK cells isolated from MS/CIS patients was due to a change in their phenotype, considering the possibility that CD56^{bright} NK cells could include a fraction of activated CD56^{dim} NK cells characterized by an up-regulation of this marker [23]. Therefore, we evaluated other typical markers defining the phenotype of CD56^{bright} NK cells in MS/CIS patients and HS. Expression of NKG2A, KIRs and CD16, as well as of the activation markers CD25 and CD69, was not different in CD56^{bright} NK cells from untreated MS/CIS patients compared to HS (Fig. 5a, Fig. 5c: gating strategy). Expression of the same markers did not differ in CD56^{dim} NK cells from MS/CIS and HS (data not shown).

We subsequently checked by flow cytometry whether the defective suppressor function of CD56^{bright} NK cells from MS/CIS subjects was due to a decreased expression of NCRs. Not only did we not observe a decreased expression of NCRs, but we found an increased expression of NKp30 and NKp46 in CD56^{bright} NK cells from untreated MS/CIS patients (Fig. 5b). Transcription of the granzyme B gene was not different in untreated MS/CIS compared to HS (Fig. 5d).

3.6. Increased expression of HLA-E on CD4^{+} T cells inhibit the regulatory function of CD56^{bright} NK cells in MS

As no significant difference was detected in the NCR/granzyme B

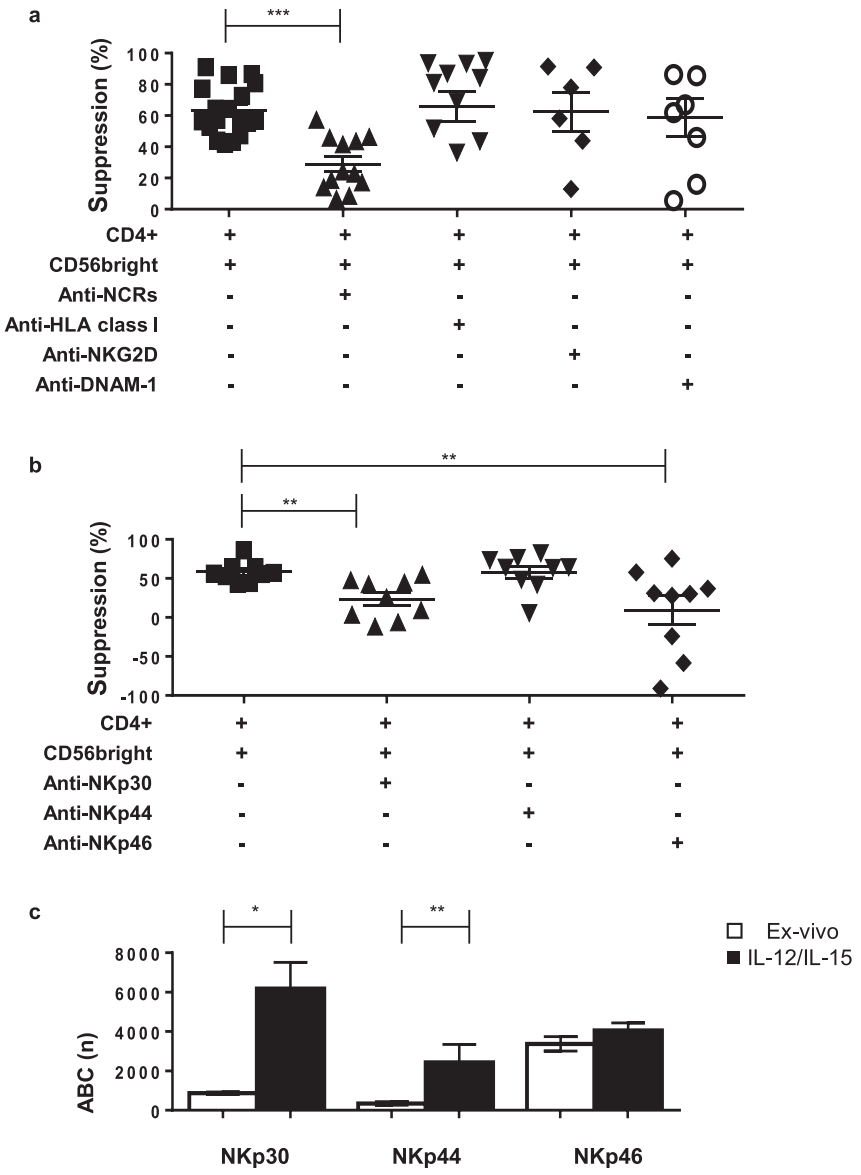

Fig. 2. The activating receptors NKp30 and NKp46 are required for the suppressive function of CD56^{bright} NK cells. a. Proliferation assays in presence of antibodies masking the activating NK receptors on CD56^{bright} NK cells (NKG2D, NCRs: NKp30, NKp44, NKp46, DNAM-1) or blocking HLA class I molecules on T cells. Every plot depicts proliferation of cells from one subject. Mean and SEM are also displayed in the graph. Wilcoxon test, $P = 0.0005$ for suppression in presence of anti-NCRs; no significant differences with other blocking antibodies. N = 6–12 healthy subjects. b. Proliferation assays in presence of antibodies masking single NCRs: NKp30, NKp44, NKp46. Significant decrease of proliferation when blocking NKp30 and NKp46 (Wilcoxon test, $P = 0.007$ for both; N = 9 subjects). c. The three activating receptors NKp30, NKp44, NKp46 were evaluated by flow cytometry on basal conditions (ex vivo) isolated and after stimulus with IL-12 and IL-15 CD56^{bright} NK cells. The number of antibodies bound per cell (ABC) was estimated adding BD quantiBRITE PE beads. Mann-Whitney test, $P = 0.02$ for NKp30, $P = 0.008$ for NKp44, P=NS for NKp46. N = 16 subjects for ex-vivo isolated CD56^{bright} NK cells, 3 subjects for stimulated CD56^{bright} NK cells.

axis of CD56^{bright} NK cells from MS/CIS patients compared to HS, we assessed whether defective suppression could be related to the T cells themselves being less prone to NK attack. Therefore, we addressed the possibility that this could be due to an increased inhibitory signal generated in NK cells upon interaction with autologus CD4^{+} T cells. We found that blocking HLA class I on T cells restored normal suppressive function of CD56^{bright} NK cells in untreated CIS/MS (Fig. 6a). CD56^{bright} NK cells sense HLA class I molecules through the inhibitory receptor NKG2A, which binds to HLA-E [24]. We did not observe differences in the expression of NKG2A among CD56^{bright} NK cells from MS/CIS and from HS (Fig. 5a); in contrast, we found an increased expression of HLA-E on T cells of subjects with MS/CIS compared to HS (Fig. 6b).

4. Discussion

The results of this work shed light on the function of the CD56^{bright} NK cell subset in healthy conditions and in MS. First, we demonstrated that CD56^{bright} NK cells are a regulatory population controlling proliferation of CD4^{+} T cells through a cytotoxic mechanism. Second, we provided evidence that such regulatory mechanism is impaired in MS/CIS.

Immune cells with regulatory function have been identified within the T- and B- compartments of the adaptive immune system and comprise, among others, natural Tregs, induced Tregs, type 1 regulatory T (Tr1) cells, and Bregs. These cell types control T effector cells through different mechanism including cytotoxicity and release of cytokines; a defect in their function is believed to

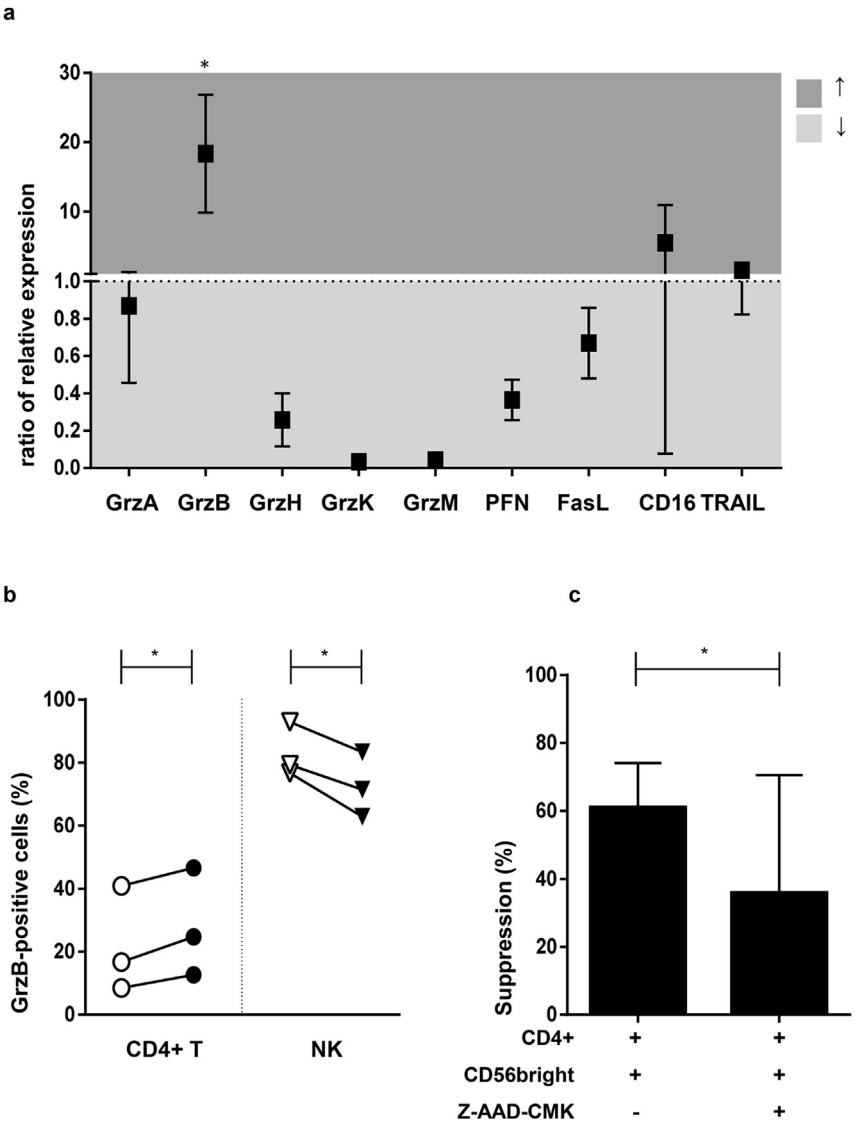

Fig. 3. Granzyme B mediates the regulatory function of CD56^{bright} NK cells. a. Real time PCR experiments showing increased (dark grey) of decreased (light grey) transcription of genes related to the lytic machinery of NK cells after 3 day-stimulus with IL-12 + IL-15. Results are shown as ratio of relative (ref gene: GAPDH) expression in stimulated CD56^{bright} NK cells compared to unstimulated CD56^{bright} NK cells. Wilcoxon test, $P = 0.03$ for granzyme B. Results are shown as mean ± SEM of at least 4 healthy subjects. b. Intracellular content of granzyme B in CD4^{+} cells cultured alone or with CD56^{bright} NK cells and in CD56^{bright} NK cells cultured alone or in presence of CD4^{+} T cells, as measured by flow cytometry. Empty symbols: culture of CD4^{+} T cells (circle) or CD56^{bright} NK cells (triangle) alone. Filled symbols: intracellular content of granzyme B in T cells (circle) and CD56^{bright} NK cells (triangle) in coculture. Paired *t*-test, $P = 0.03$ for CD4^{+} cells and CD56^{bright} NK cells. N = 3 healthy subjects. c. Suppression of activated CD4^{+} T cell proliferation in absence or presence of the granzyme B inhibitor Z-AAD-CMK. Wilcoxon test, $P = 0.046$. N = 7 healthy subjects.

contribute to autoimmunity. Regulatory cell populations among the innate immune system, and particularly within the ILC family, are much less characterized. We focused on CD56^{bright} NK cells because an increased number of this NK cell subset has been observed in MS patients following successful treatments, for example with daclizumab [25], and in pregnancy, a physiological condition often associated with MS disease remission [13]. Therefore, we hypothesized that these cells may contribute to maintaining immune homeostasis and might be altered in an autoimmune disease such as MS. CD56^{bright} NK cells had been previously marked as "immunoregulatory" due to their capability of releasing large amounts of cytokines, their lower cytotoxic potential towards cancer and virus-infected cells and their enrichment in lymph nodes T cell area. Others have previously shown that CD56^{bright} NK cells can kill pre-activated T cells [26–28]. Compared to other studies, we chose to assess the impact of CD56^{bright} NK cells on the proliferation of autologous CD4^{+} T cells. Therefore, instead of performing a short lasting cytotoxicity assay with pre-activated CD56^{bright} NK cells and T cells, we cultured CD56^{bright} NK cells and ex-vivo isolated autologous CD4^{+} T cells in presence of T-cell stimulus and observed that the presence of CD56^{bright} NK cells markedly decreased autologous CD4+T cell proliferation in HS. This effect was dependent on CD56^{bright} NK-mediated cytotoxicity against autologous T cells undergoing activation and may indicate that the role of CD56^{bright} NK cells is to prevent excessive activation of CD4^{+} T cells. At this stage, we do not know whether this effect targets specific subsets of CD4^{+} T cells including Tregs, effector cells and possibly autoreactive cells.

We found that two activating NK receptors, the NCRs NKp30 and NKp46, mediate the recognition and killing of autologous T cells by CD56^{bright} NK cells. Some NCRs ligands are unknown at this time [29]; in this context our study suggests that ligands for such

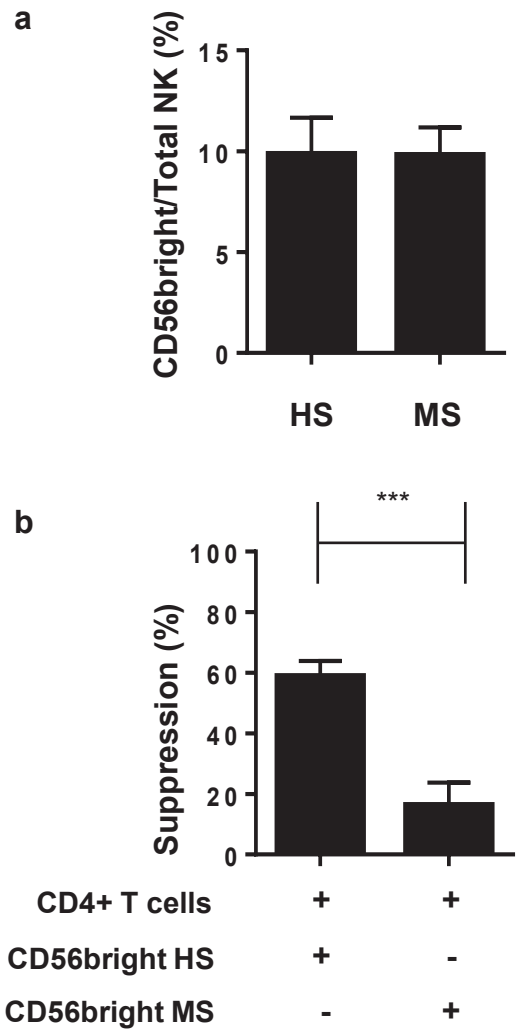

Fig. 4. CD56^{bright} NK cells from untreated MS/CIS patients do not suppress autologous T cell proliferation. a. Mean ± SD of the proportion of CD56^{bright} NK cells over total NK cells in untreated MS/CIS patients compared to HS, as evaluated by flow cytometry in negatively isolated NK cells. Wilcoxon test, P = ns. N = 12 HS and 13 MS/CIS patients. b. Suppressive function of CD56^{bright} NK cells from untreated MS/CIS patients towards autologous CD4^{+} T cells compared to suppressive function of CD56^{bright} NK cells towards autologous CD4^{+} T cells from HS, in paired experiments. Wilcoxon test, P = 0.0002. N = 13 subjects with CIS or MS and 13 paired HS.

receptors are expressed by autologous T cells undergoing activation, thus providing a possible mechanism to maintain homeostasis in healthy subjects by modulating any excess of activation. Moreover, upon IL-12 and IL-15 stimulation, we observed a significant upregulation of granzyme B in CD56^{bright} NK cells, whose blockade resulted in a significant decrease of suppression of autologous T cell proliferation. These data suggest that in autologous activated CD4^{+} T cells/CD56^{bright} NK cells cocultures, after NCRs engagement, a transfer of granzyme B from NK cells to T cells may be responsible for NK-mediated T cell death. Interestingly, killing by granzyme B was described by Tr1 cells, that appear similar to CD56^{bright} NK cells in other aspects: they simultaneously secrete IFN-gamma and IL-10 [17,30] and may be induced by IL-27 [9,31].

In HS, the proinflammatory cytokine IL-12, which promotes activation of the regulatory CD56^{bright} NK cells in combination with IL-15, has been shown to dampen the regulatory function of FOXP3+ T regulatory cells [32]. Therefore, we may speculate that, when inflammation occurs in HS, IL-12 is produced, and Tregs are less functional, CD56^{bright} NK cells represent a main regulatory subset whose role may be important to prevent excess of T cell response. If this is the case, a defect in CD56^{bright} NK cells-mediated immunoregulation may be associated to the development of uncontrolled T cells responses and contribute to autoimmunity.

Accordingly, we found that in early MS/CIS subjects, CD56^{bright} NK cells suppressed CD4^{+} T cells proliferation less efficiently than in HS despite similar proportion of CD56^{bright} NK cells over total NK cells. Defective function of CD56^{bright} NK cells in MS/CIS was not explained by higher baseline activation, as shown by comparable expression of activation markers and production of granzyme B with respect to NK cells from HS. Neither have we found a decreased expression of NCRs involved in the suppressive function; these were indeed upregulated, in what may be an attempt to compensate for the lower suppressive function.

Conversely, we observed that blocking the inhibitory interaction between the NKG2A receptor expressed by CD56^{bright} NK cells and its ligand HLA-E, whose surface expression on CD4^{+} T cells was found higher in MS subjects compared to HS, recovers CD56^{bright} NK cells ability to suppress T cell proliferation in MS/CIS. This suggests that impairment of the regulatory function of CD56^{bright} NK cells is due to an excess of HLA-E-mediated inhibition by activated CD4^{+} T cells from MS/CIS patients. Interestingly, in line with this assumption, upregulation of HLA-E in active MS lesions and in the CSF of MS patients has been observed [33,34]. All together these findings may suggest an impairment of CD56^{bright} NK cells regulatory functions not only in peripheral blood, but also within the CNS of MS subjects.

Studies in the mouse model for MS, experimental autoimmune encephalomyelitis (EAE), have provided conflicting results on the role, beneficial vs. detrimental, of NK cells in disease, as reviewed in Ref. [35]. This may be related to the differences among animal and human diseases, and to the difficulty to compare human and mouse NK cell subsets: since mice NK cells do not express CD56, the counterpart of CD56^{bright} NK cells in mice has not been identified. Moreover, mouse NK cells express an analogue of NKp46 as the only NCR. With these premises, in mice, a regulatory role for NK cells expressing NKG2A has been observed by Lu and coauthors; they showed that blocking the interaction between NKG2A and the murine analogue of HLA-E on T cells, Qa-1^{b}/Qdm, enables NK cells to control T cell activation, thereby ameliorating EAE [36], and is associated with lysis of pathogenic T cells and decreased infiltration of T cells into the CNS [37]. Others have found a NKG2D-mediated killing of T cells by NK cells in mice [38]. In humans, Nielsen and coauthors [26] observed cytotoxicity by CD56^{bright} NK cells towards activated CD4^{+} T cells, mainly mediated by the engagement of NKG2D, LFA-1 and TRAIL. The authors also found that blocking NKp46 decreased degranulation of CD56^{bright} NK cells in presence of activated CD4^{+} T cells. In the study by Nielsen and coauthors, CD56^{bright} NK cells were not purified by cell sorting but gated among total NK cells cultured in presence of target T cells. Others have found a cytotoxic mechanism of polyclonally activated NK cells towards T cells, again relying on NKG2D [27]. Schlums and coauthors recently described cytotoxicity of the CD56^{dim} NK cell subset towards autologous CD4^{+} T cells related to the expression of receptors belonging to the signaling lymphocytic activation molecule (SLAM) family [28]. Different from these studies, we sorted CD56^{bright} NK cells before cytokine-mediated stimulation. Thus we avoided the cytokine-induced NK cell activation which is known to result in upregulation of the CD56 marker by CD56^{dim} NK cells [23]. On the other side, the thorough two-step isolation method of a cell population which is scarce in blood (about 1% of circulating lymphocytes) provided us with low numbers of cells for experimental conditions and limited the possibility to assess degranulation of CD56^{bright} NK cells at multiple time-points during the NK-T

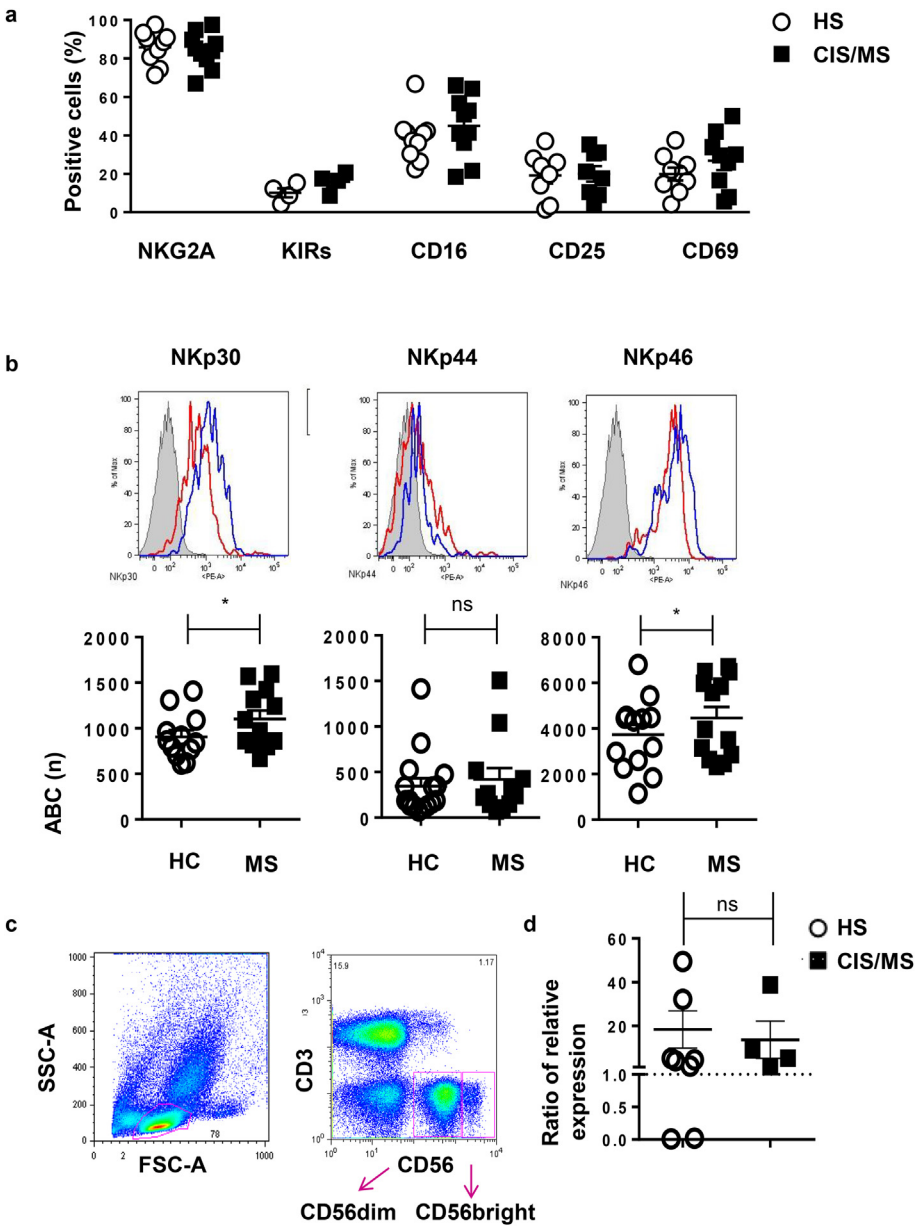

Fig. 5. Analysis for relevant cell surface molecules indicate that CD56^{bright} NK cells from untreated MS/CIS patients and HS have similar phenotype and produce granzyme B. a. FACS analysis for specific NK cell markers in CD56^{bright} NK cells from HS and untreated MS/CIS. Cells were gated from total PBMCs as CD3negative/CD56^{bright}. Results are shown as scatter dot plots, mean ± SEM. N = 4–10 HS and 4–10 untreated paired MS/CIS patients. HS vs. MS/CIS, Wilcoxon test, *P*= NS for all markers. b. Expression of the NCRs NKp30, NKp44 and NKp46 in CD56^{bright} NK cells from untreated MS/CIS patients compared to HS. The number of receptors per cell was assessed through flow cytometry with the BD QuantiBRITE method. Top panels show FACS histograms from one representative experiment, with blue histogram showing the mean fluorescence intensity of the receptor in CD56^{bright} NK cells from untreated MS/CIS patient, and red histogram depicting data of the paired HS. The grey-shaded histogram represents the negative control (staining for NKp44 in CD56^{dim} NK cells). Bottom panels show expression of the NCRs from 12 paired HS and untreated MS/CIS patients (NKp30, NKp44), and 13 paired HS and CIS/MS patients (NKp46). Wilcoxon test, $P = 0.02$ for NKp30 and $P = 0.04$ for NKp46. c. Gating strategy for cytometric analysis of NK cell subsets. d. Real-time PCR data showing expression of granzyme B in CD56^{bright} NK cells isolated from 9 HS and 4 untreated MS/CIS patients after 24 h of activation with IL-12 and IL-15 compared to CD56^{bright} NK cells cultured for 24 h without stimulus. Mann-Whitney test, P = NS.

coculture. This, and the fact that it is conceivable that killing by CD56^{bright} NK cells continuously occurs over time during the culture, may explain, in our opinion, the lower degree of NK degranulation we observed compared to other studies.

The evidence that the mechanism of action of daclizumab relies, at least in part, on the expansion of CD56^{bright} NK cells had pointed towards a beneficial role of this cell subset. This work shows for the first time an impairment in NK cell-mediated immunoregulation in untreated MS, that is attributable more to the T cells being resistant to NK suppressive action than to intrinsic defects of the NK cells themselves. To confirm the parallelism between CD56^{bright} NK cells and regulatory cells, others showed that in MS, CD4^{+} T cells escape from regulation by Tregs [39].

Increasing the number of CD56^{bright} NK cells, a major mechanism of action of daclizumab in MS, appears to overcome this defect. This is in line with what has been reported by the group of Bielekova, which showed that daclizumab, in addition to increasing the number of circulating CD56^{bright} NK cells, changes their phenotype in order to make them more cytotoxic [14,16]; we cannot exclude that daclizumab affects also CD4^{+} T cells, by making

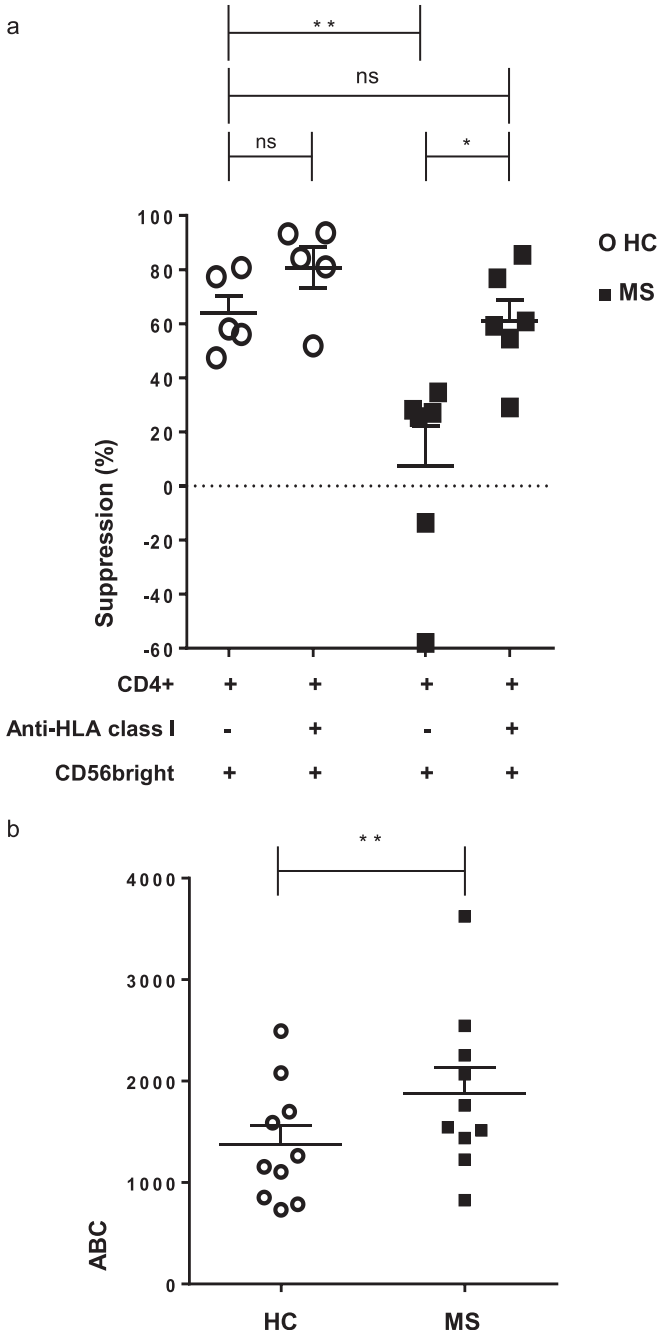

Fig. 6. Increased HLA-E on CD4$^+$ T cells inhibits cytotoxicity of CD56^{bright} NK cells from untreated CIS/MS patients. a. Suppression of autologous CD4$^+$ T cells proliferation by CD56^{bright} NK cells from HS or untreated MS/CIS patients in absence or presence of anti-HLA I antibody (paired experiments with at least one HS and one MS/CIS patient). N = 5 HS, 6 MS/CIS (without anti-HLA I antibody) and 5 MS/CIS patients (with anti-HLA I antibody). Mann-Whitney test, P = 0.004 for HS vs MS/CIS (both in absence of anti-HLA class I antibody); P = ns for HS in absence of anti-HLA class I antibody vs MS/CIS in presence of anti-HLA class I antibody, P = 0.004 for MS/CIS patients in absence vs CIS/MS patients in presence of anti-HLA class I antibody. b. Expression of HLA-E (ligand for NKG2A) on CD4$^+$ T cells of untreated MS/CIS patients compared to HS, measured by flow cytometry with the BD QuantiBRITE method. ABC: number of antibodies bound per cell. N = 10 HS and 10 subjects with MS/CIS.

them more susceptible to NK cells. However, some patients treated with daclizumab have experienced adverse events possibly associated to accumulation of CD56$^+$ cells in lymph nodes or in the breasts [40]. The findings of the present study provide new insights about the role of CD56^{bright} NK cells in the regulation of immune responses and suggest that reverting defective immunoregulation exerted by CD56^{bright} NK cells may be as well a potential therapeutic target in MS.

In conclusion, the results of our study show the possible importance of innate immunity resident in lymph nodes in preventing autoimmune responses by controlling autologous T cell activation, and suggests that the evasion of T cells from CD56^{bright} NK cell control may contribute to the deregulated T cell response observed in MS.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2016.04.003.

Conflict of interest statement

AL received honoraria for speaking by Biogen and Novartis, consulting fees by Merck Serono, Sanofi-Genzyme, and Novartis and funding for travel from Teva, Merck Serono, Biogen, Novartis. EA declares no conflicts of interest. NKDR declares no conflicts of interest. FI declares no conflicts of interest. EM declares no conflicts of interest. SS declares no conflicts of interest. RG received funding for research from Novartis, Biogen and EMD Serono. HLW has received honoraria for consulting from Teva, Biogen, Novartis, Therapix, Serono and Genzyme; he has received funding for research from Serono, Teva, Biogen and Genzyme. AM is a founder and shareholder of Innate-Pharma (Marseille, France). GLM has received honoraria, travel expenses, and financial support for research from Bayer Schering, Biogen Idec, Sanofi Aventis, Teva, Merck Serono and Novartis. AU has received consulting honoraria and/or speaker fees from Biogen, Genzyme, Roche, Merck Serono, TEVA and Novartis and financial support for research from Biogen and Merck Serono.

Author contributions

AL designed the study, performed experiments, analyzed data and wrote the manuscript. EA performed experiments and analyzed data. NKDR assisted with design of the experiments, discussed/interpreted data and edited the manuscript. FI performed experiments and sorted cells by flow cytometry. EM, SS and AM assisted with design of experiments, provided reagents, discussed/interpreted data and edited the manuscript. RG performed experiments. HLW, GLM discussed/interpreted data. AU supervised the study and edited the manuscript.

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