International Immunology, Vol. 23, No. 2, pp. 139–148
doi:10.1093/intimm/dxq463
Advance Access publication 6 January 2011

Impaired IFN-γ production and proliferation of NK cells in Multiple Sclerosis

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Received 10 November 2009, *accepted* 29 November 2010

Abstract

NK cells are multicompetent lymphocytes of the innate immune system with a central role in host defense and immune regulation. Studies in experimental animal models of multiple sclerosis (MS) provided evidence for both pathologic and protective effects of NK cells. Humans harbor two functionally distinct NK-cell subsets exerting either predominantly cytotoxic ($CD56^{dim}CD16^{+}$) or immunoregulatory ($CD56^{bright}CD16^{-}$) functions. We analyzed these two subsets and their functions in the peripheral blood of untreated patients with relapsing-remitting MS compared with healthy blood donors. While *ex vivo* frequencies of $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells were similar in patients and controls, we found that cytokine-driven *in vitro* accumulation and IFN-γ production of $CD56^{bright}CD16^{-}$ NK cells but not of their $CD56^{dim}CD16^{+}$ counterparts were substantially diminished in MS. Impaired expansion of $CD56^{bright}CD16^{-}$ NK cells was cell intrinsic because the observed effects could be reproduced with purified NK cells in an independent cohort of patients and controls. In contrast, cytolytic NK-cell activity toward the human erythromyeloblastoid leukemia cell line K562, the allogeneic $CD4^{+}$ T cell line CEM and allogeneic primary $CD4^{+}$ T-cell blasts was unchanged. Thus, characteristic functions of $CD56^{bright}CD16^{-}$ NK cells, namely cytokine-induced NK cell expansion and IFN-γ production, are compromised in the NK cell compartment of MS patients.

Keywords: NK cell, multiple sclerosis, autoimmunity

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system, which is believed to be initiated and mediated by autoreactive T cells (1, 2). Perturbations in immunomodulatory networks that include T_h2 cells, regulatory T cells, NK cells and others are thought to be in part responsible for the relapsing–remitting or chronic progressive nature of the disease (3, 4).

NK cells are multicompetent innate lymphocytes that originate from bone marrow precursors but can mature in a variety of primary and secondary lymphoid tissues (5–7). Access to these tissues and interaction with dendritic cells (DCs) and their cytokines (i.e. IL-12 and IL-15) seems to be required for optimal NK cell activation (8–10). The two key effector functions of NK cells—killing and cytokine production—are in humans mediated by two main subsets of these innate lymphocytes that can be differentiated from each other based on the expression of CD16 and CD56 (10, 11). $CD56^{dim}CD16^{+}$ NK cells constitute about 90% of total blood NK cells, efficiently kill target cells and secrete only low levels of IFN-γ. In contrast, $CD56^{bright}CD16^{-}$ NK cells constitute <10% of total blood NK cells but are enriched in secondary lymphoid tissues (11, 12), sites of autoimmune inflammation (13) and tumors (14). $CD56^{bright}CD16^{-}$ cells produce a large amount of cytokines upon stimulation but acquire cytolytic activity only after prolonged activation (15).

In this study, we explored frequencies and effector functions of $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ blood NK cells in untreated patients with relapsing-remitting MS compared with healthy individuals. Our data indicate that the capability to produce IFN-γ and to proliferate following cytokine activation is impaired in NK cells of MS patients in general,

whereas cytolytic NK cell function is unchanged in MS. Since the impaired functions are primarily mediated by $CD56^{bright}CD16^{-}$ NK cells and their expansion and IFN-γ production is mainly compromised, this NK cell subset seems to be primarily deficient in MS patients.

Methods

Patients

Twenty treatment-naive patients with clinically definite relapsing-remitting MS fulfilling the revised 'McDonald' criteria for diagnosing MS (16, 17) and 25 healthy donors (HDs) were enrolled in the New York cohort (Table 1). Patients were recruited at the Corinne Goldsmith Dickinson Center for Multiple Sclerosis, Mount Sinai School of Medicine (New York, NY) and the Judith Jaffe Multiple Sclerosis Center, Weill Cornell Medical College (New York, NY). Healthy controls were recruited at the New York blood bank. Twelve patients with clinically definite relapsing-remitting MS fulfilling the revised McDonald criteria above who were treatment naive for disease modifying drugs and were at the time of sampling at least 8 weeks after their last steroid treatment were enrolled in the Marburg cohort (Table 2). Twelve HDs were recruited through the Zürich blood bank. The study was approved by the local Institutional Review Boards (Institutional Review Board of the Rockefeller University, Mount Sinai School of Medicine, Weill Cornell Medical College and University of Marburg) and all subjects provided informed consent.

Cell culture

PBMCs were isolated from blood samples via density centrifugation. PBMCs were either directly stained with monoclonal antibodies or cultured for 24 and 72 h in the presence of 100 U ml^{-1} human recombinant (hr) IL-2 (Peprotech, Rocky Hill, NJ, USA), 0.5 ng ml^{-1} hrIL-12 (Peprotech) or without addition of cytokines. For some experiments, NK cells were isolated by negative selection using the NK cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the isolated NK cells was higher than 95% and contained less than 5% contaminating T cells as determined by flow cytometry.

Proliferation assay by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution

Purified NK cells were labeled with 5 μM CFSE in PBS for 10 min at 37°C. After washing twice with PBS, cells were cultured for 6 days at 37°C with 0.5 ng ml^{-1} hrIL-12 (Peprotech). CFSE fluorescence was evaluated on $CD3^{-}CD56^{+}$ cells by flow cytometry.

Flow cytometry

PBMC were washed in PBS and stained with the indicated combinations of directly fluorochrome-labeled antibodies against CD3, CD16, CD25, CD56, CD69, NKG2D, NKp44, NKG2A, HLA-DR, CXCR1, IFN-γ and a respective isotype control (Invitrogen, Carlsbad, CA, USA) as well as IL-15 receptor alpha (IL-15Rα) and a respective isotype control (R&D, Minneapolis, MN, USA) for 30 min at 4°C. Cells stained for IFN-γ were fixed and permeabilized using the BD Cytofix/Cytoperm™ Kit (BD, Franklin Lakes, NJ, USA) according to the manufacturer's recommendations. The cells were washed once with PBS prior to FACS analysis. The samples were measured on a BD LSR II or a FACS Canto II flow cytometer. Gating and calculations for precursor frequencies were performed with FlowJo software.

Cytotoxicity assay

Cytolytic NK cell activity toward susceptible target cells [the $CD4^{+}$ T cell line (CEM), which constitutively expresses the NKG2D ligand MHC class I-related chain (MIC) A, primary allogeneic T cell blasts and the human erythromyeloblastoid

Table 1. Patients and Controls (New York cohort)

	Multiple sclerosis	HD
Number	20	25
Age (mean, range)	33.3 (18–52)	na
Sex ratio (female to male)	16:4	na
Relapsing-remitting disease course	20 (100%)	na
Disease duration at time of samples (mean, range in years)	5.4 (0.5–20)	na
EDSS score (mean, range in years)	1.2 (0–4)	na
Total number of attacks in first 2 years (mean, range)	1.7 (0–5)	na
Number of Gd+ lesions (mean, range)	0.7 (0–5)	na
Number of T2 lesions (mean, range)	5.8 (1–17)	na
Number of T1 lesions (mean, range)	0.5 (0–2)	na
Treatment at time of sample	0/20 (0%)	na

Table 2. Patients and controls (Marburg cohort)

	Multiple sclerosis	HD
Number	12	12
Age (mean ± SD, range)	32.8 ±10.2 (19–53)	na
Sex ratio (female to male)	5:1	na
Relapsing-remitting disease course	12 (100%)	na
Disease duration at time of sampling (mean ± SD, range in months)	32.6 ± 9.6 (24–51)	na
EDSS score (mean ± SD, range)	1.4 ± 0.6 (1.0–2.5)	na
Total number of attacks in first 2 years (mean ± SD, range)	2.7 ± 1.0 (2–5)	na
Number of Gd+ lesions cMRI at diagnosis (mean ± SD, range)	0.5 ± 0.9 (0–3)	na
Number of T2 lesions cMRI (mean ± SD, range)	7.9 ± 4.5 (3–17)	na
Number of T1 lesions cMRI (mean ± SD, range)	1.4 ± 1.3 (0–4)	na
Treatment at time of sampling	0/12 (0%)	na

leukemia cell line K562] was quantified using a flow cytometry-based cytotoxicity assay as previously described (18). The fluorophore PKH-26 (Sigma) was used to label target cells and the DNA-intercalating dye TO-PRO-3 iodide (TP3; Invitrogen) to determine the viability of targeted PHK-26$^+$ cells upon short-term coculture with NK cells. The extent of cytotoxicity was quantified by the relative number of live cells, labeled with PKH-26, and dead cells, labeled with both PKH-26 and TP3 (18, 19). Target cells were cocultured with unstimulated as well as IL-2- and IL-12-stimulated PBMCs at a ratio of 1:100 to achieve an NK cell effector to target cell ratio of 10:1 or purified NK cells at a effector to target cell ratio of 10:1. Effector and target cells were coincubated for 4 h to assess rapid primarily perforin-mediated cytotoxicity of NK cells (20, 21).

Statistics

Frequencies of circulating mononuclear blood cells, of protein expression levels as defined by mean fluorescent intensities and of TP3$^+$ target cells, were compared using the non-parametric Mann–Whitney *U*-test and statistic significance was judged after correction for multiple testing (Bonferroni correction). Statistical significance was indicated in the figures by asterisks with the *P*-values described in the figure legends.

Results

Impaired accumulation of cytokine-activated NK cells in MS

To investigate NK cell functions in MS, we first determined the frequencies of circulating CD3$^-$CD56^{dim}CD16$^+$ and CD3$^-$CD56^{bright}CD16$^-$ NK cells and CD3$^+$ T cells in 20 untreated patients with relapsing-remitting MS compared with 25 healthy blood donors (Table 1). We found that *ex vivo* frequencies of both NK cell subsets were unchanged in MS (mean frequency in percent of CD3$^-$ lymphocytes ± SEM in HD versus MS: 20.8 ± 2.6 versus 18.8 ± 1.7 for CD56^{dim}CD16$^+$ NK cells and 2.2 ± 0.2 versus 2.1 ± 0.3 for CD56^{bright} CD16$^-$ NK cells; Fig. 1B). Gating on total

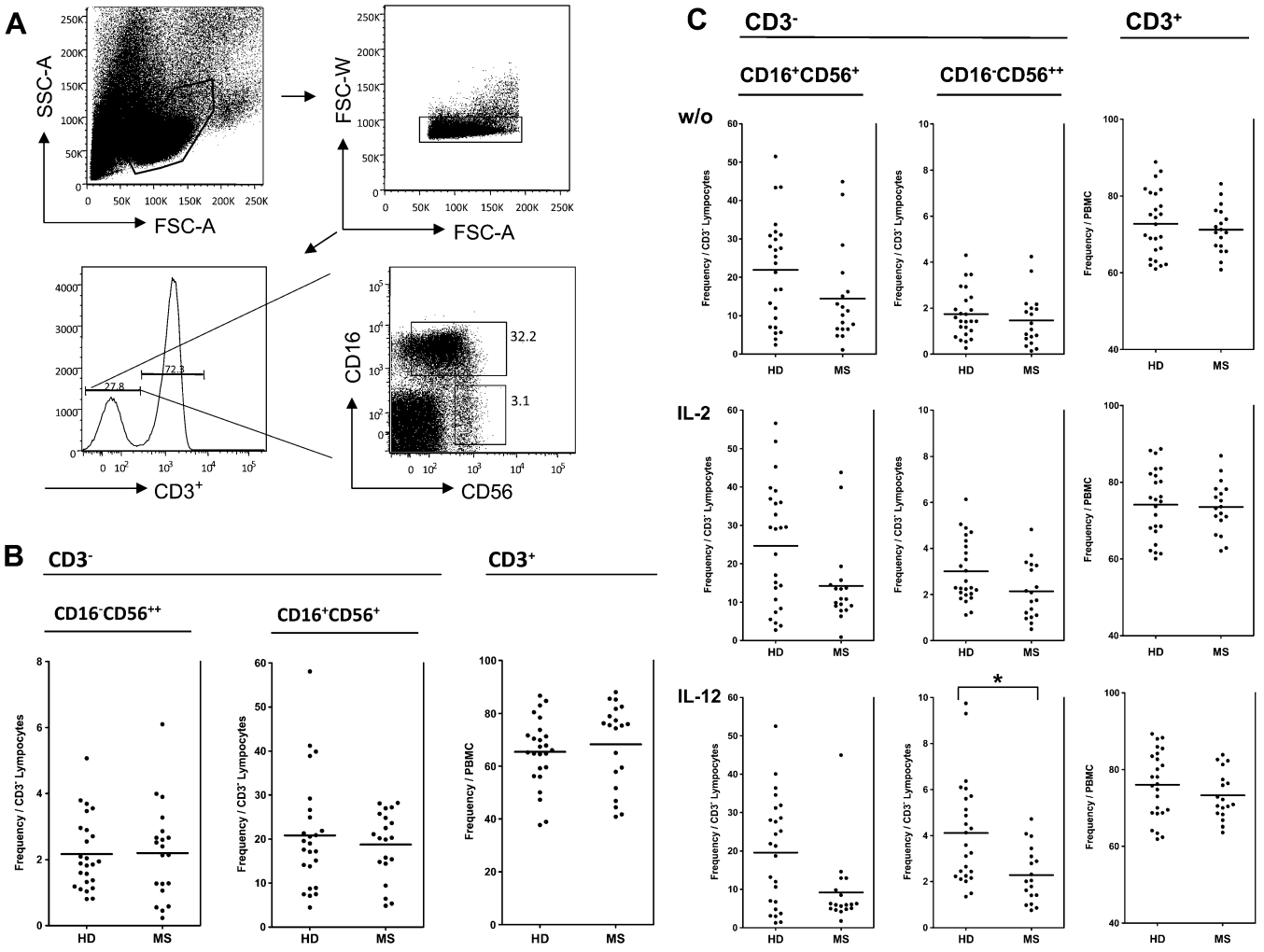

Fig. 1. Impaired accumulation of CD3$^-$CD56^{bright}CD16$^-$ NK cells compared with CD3$^-$CD56^{dim}CD16$^+$ NK cells and CD3$^+$ T cells in MS patients. (A) The gating strategy leading to the composite data in (B) and (C) is shown for one representative healthy donor (HD). (B) *Ex vivo* frequencies of NK cell subsets did not vary significantly between HDs and MS patients. (C) PBMCs were stimulated with cytokines IL-2 (100 U ml^{-1}) or IL-12 (0.5 ng ml^{-1}) or left unstimulated without for 72 h and percentages of CD3$^-$CD56^{dim}CD16$^+$ NK, CD3$^-$CD56^{bright}CD16$^-$ NK and CD3$^+$ T cells were determined. Twenty MS patients and 25 HDs are summarized. Asterisks indicate statistical significance (*P = 0.017).

leucocytes and relating the frequencies either to total leucocytes or $CD3^-$ lymphocytes produced similar results (Supplementary Figure 1 is available at *International Immunology* Online). In order to study the ability of patients' NK cells to expand, we next exposed PBMCs to NK cell-activating cytokines, i.e. IL-2 (100 U ml^{-1}) as classical NK cell mitogen and IL-12 (0.5 ng ml^{-1}) as NK cell-activating monokine, and determined NK cell and T-cell frequencies after 72 h of *in vitro* culture. Limiting amounts of IL-12 were chosen to mimic DC-induced NK cell accumulation and IFN-γ secretion (8, 10). CD16 was not down-regulated significantly in sorted NK cell subsets with and without cytokines over 72 h under our experimental conditions (Supplementary Figure 2 is available at *International Immunology* Online). As demonstrated before (10), $CD56^{bright}CD16^-$ NK cells preferentially expand in response to IL-12 treatment. Under these conditions, we found that NK cells from MS patients differed from those derived from healthy controls in their capacity to accumulate in response to activating cytokines (Fig. 1). In accordance with the NK cell subset preference of IL-12 stimulation, accumulation of $CD3^-CD56^{bright}CD16^-$ NK cells from MS patients was impaired in response to this cytokine (34% reduction compared with mean frequencies in HD; $P = 0.003$). Furthermore, accumulation of IL-2-stimulated $CD56^{bright}CD16^-$ NK cells ($P = 0.04$) and IL-12-stimulated $CD56^{dim}CD16^+$ NK cells ($P = 0.03$) tended to be lower in MS patients. However, only the aforementioned differences did reach statistical significance following Bonferroni correction (alpha = 0.017), suggesting a predominant impairment of IL-12-mediated $CD56^{bright}CD16^-$ NK cell accumulation in MS. Frequencies of $CD3^+$ T cells from patients and controls were similar in cultures treated with and without cytokines and cultures from MS patients did not differ significantly from those derived from controls in total numbers of live cells as determined by trypan-blue staining after 72 h of culture (data not shown). These data indicate an impairment of $CD56^{bright}CD16^-$ NK cells from MS patients to accumulate in response to the NK cell activating monokine IL-12.

Phenotype of blood NK cells in MS indicates increased in vivo activation

We next determined the expression profile of inhibitory (NKG2A) and stimulatory NK cell receptors (NKG2D and NKp44) as well as surface markers indicative of activation (CD25, CD69 and HLA-DR) and homing to inflammatory sites (CXCR1) on unstimulated and cytokine-treated NK cells (Bonferroni correction, alpha = 0.007). Frequencies of both $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ NK cells expressing HLA-DR ($P = 0.008$ and $P = 0.02$, respectively) and CD69 ($P = 0.12$ and $P = 0.03$, respectively) tended to be increased in untreated cultures from patients with MS (Fig. 2). Similar tendencies could be observed following IL-2 stimulation for $CD56^{bright}$ and $CD56^{dim}$ NK cells expressing HLA-DR ($P = 0.01$ and $P = 0.01$, respectively) and CD69 ($P = 0.82$ and $P = 0.001$, respectively). IL-12 stimulation led to an expansion of $HLA\text{-}DR^+$ and $CD69^+$ NK cells in healthy controls but showed only minimal effects in MS patients, resulting in a loss of differences between both groups. Following correction for multiple testing, we found that only CD69-expressing $CD56^{dim}CD16^+$ NK cells were moderately increased in frequency in IL-2-treated cultures from MS patients. Frequencies of NK cells expressing NKG2A, NKG2D, NKp44, CD25 and CXCR1 as well as of T cells expressing these markers including HLA-DR and CD69 were similar in patients and controls in all conditions tested (data not shown). Thus, impaired accumulation of $CD56^{bright}CD16^-$ NK cells is associated with moderately higher frequencies of $CD69^+CD56^{dim}CD16^+$ NK cells, suggesting slightly increased *in vivo* activation of these cells in MS.

Impaired IFN-γ production by $CD56^{bright}CD16^-$ NK cells in MS

To further investigate NK cell function in patients with MS, we determined IFN-γ production by intracellular cytokine staining after short-term restimulation of NK cells and T cells *in vitro*. As expected, significant IFN-γ production was observed only in IL-12-activated $CD56^{bright}CD16^-$ NK cells and IL-12 stimulation elicited higher frequencies of IFN-γ -positive $CD56^{bright}CD16^-$ NK cells in than IL-2 stimulation (Fig. 3). IFN-γ production by $CD56^{bright}CD16^-$ NK cells was severely diminished in MS patients (52% decrease from HD to MS upon IL-12 stimulation, $P = 0.002$). This difference remained statistically significant following Bonferroni correction (alpha = 0.017). Both $CD56^{dim}CD16^+$ NK cells, as well as T cells, produced only low to undetectable amount of IFN-γ in response to the used stimuli (Fig. 3). These data indicate that $CD56^{bright}CD16^-$ NK cells of MS patients are not only impaired in their ability to accumulate but also produce less IFN-γ than their counterparts from HDs after stimulation with IL-12.

IL-15 receptor alpha expression on NK cells and T cells in MS

We have recently shown that IL-15 receptor alpha (IL-15Rα) accumulates at the synapse between human DCs and NK cells and contributes to NK cell survival (22). Lack of IL-15Rα surface expression on NK cells and T cells has been documented in individuals with a history of infectious mononucleosis (IM) (23), a syndrome associated with a moderately increased risk to develop MS (24, 25). We therefore investigated whether poor NK cell expansions would be associated with a lack of or impaired expression of IL-15Rα in MS patients. We determined expression levels of IL-15Rα in $CD56^{bright}CD16^-$ NK cells, $CD56^{dim}CD16^+$ NK cells and $CD3^+$ T cells as well as frequencies of IL-15Rα-expressing lymphocytes. Membrane-bound IL-15Rα could be detected in both $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ NK cell subsets as well as in T cells (Supplementary Figure 3A is available at *International Immunology* Online). Expression levels of membrane-bound as well as of total IL-15Rα proteins were similar in MS patients and controls in any cell compartment studied (Supplementary Figure 3B is available at *International Immunology* Online). Furthermore, the frequency of IL-15Rα-expressing cells was similar in patients and in controls (IL-15Rα surface expression: mean frequency in percent of $CD3^-$ lymphocytes ± SEM in HD versus MS: 1.5 ± 0.2 versus 2.1 ± 0.4 for $CD56^{dim}$ $CD16^+$ NK cells and

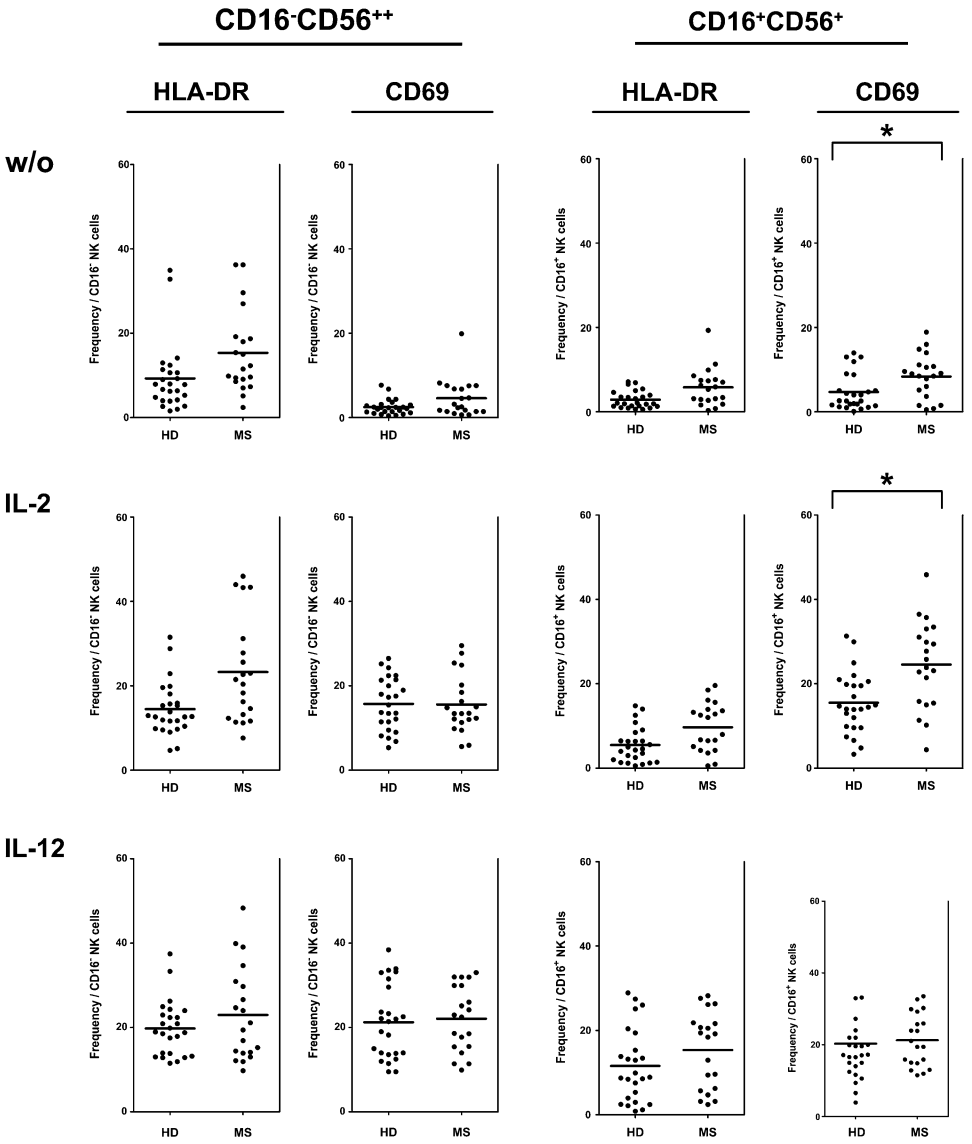

Fig. 2. Frequencies of HLA-DR$^+$ and CD69$^+$ NK cells in MS. PBMCs were stimulated with cytokines (IL-2 or IL-12) or left unstimulated without for 72 h. HLA-DR and CD69 surface expression was analyzed by flow cytometry for CD56^{bright}CD16$^-$ (left two columns) and CD56^{dim}CD16$^+$ NK cells (right two columns). Twenty MS patients and 25 healthy donors (HD) are summarized. Asterisks indicate statistical significance (*P = 0.007).

0.9 ± 0.1 versus 1.3 ± 0.2 for CD56^{bright} CD16$^-$ NK cells and 3.1 ± 0.5 versus 2.5 ± 0.4 for T cells; total IL-15Rα expression: 8.8 ± 1.4 versus 12.1 ± 2.3 for CD56^{dim} CD16$^+$ NK cells and 6.5 ± 1.0 versus 4.4 ± 0.6 for CD56^{bright} CD16$^-$ NK cells and 19.4 ± 2.0 versus 10.1 ± 1.6 for T cells). We conclude that impaired NK cell accumulation does not result from reduced expression or absence of the high-affinity IL-15Rα chain in MS.

Cytolytic NK cell function is unchanged in MS

NK cells have been proposed to mediate their immunoregulatory functions in MS and various experimental autoimmune encephalomyelitis (EAE) models via killing of encephalitogenic T cells (26–28). Previous studies have shown that human antigen-activated T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK-cell lysis (29). We therefore analyzed the cytolytic activity of NK cells in patients with MS compared with healthy blood donors using a CD4$^+$ T cell line (CEM), which constitutively expresses the NKG2D ligand MHC class I-related chain (MIC) A, primary allogeneic T cell blasts and the human erythromyeloblastoid leukemia cell line K562 as target cells. The fluorophore PKH-26 was used to label target cells and the DNA-intercalating dye TO-PRO-3 iodide (TP3) to determine the viability of targeted PHK-26$^+$ T cells upon short-term coculture with NK cells. The extent of cytotoxicity was quantified by the relative number of live T cells labeled with PKH-26 and dead permeabilized cells labeled with both PKH-26 and TP3 (18, 19) (Fig. 4). Effector and target cells were coincubated for 4 h to assess rapid primarily perforin-mediated cytotoxicity of NK cells (20, 21). The

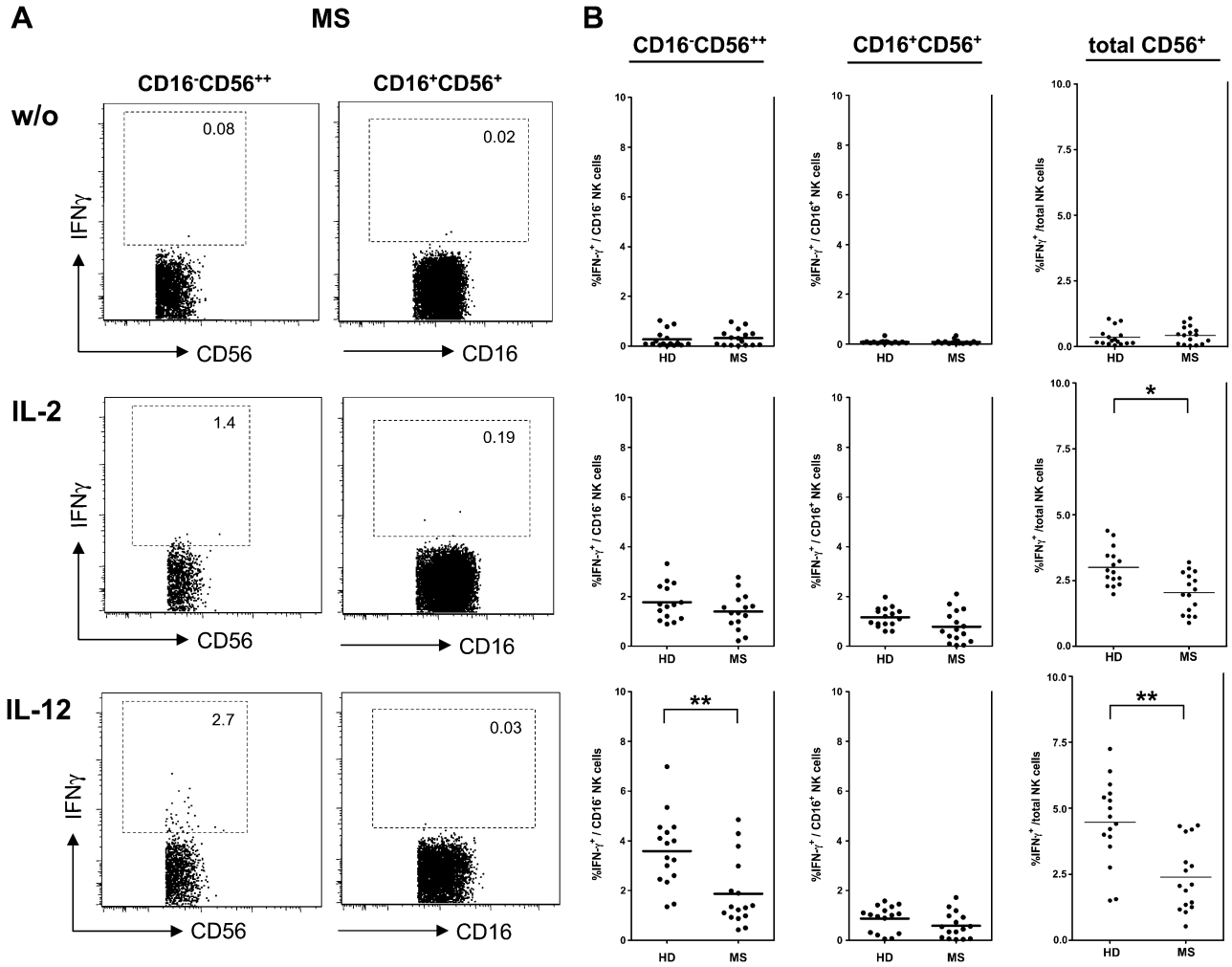

Fig. 3. Reduced IFN-γ production in IL-12-stimulated CD56^{bright}CD16^{-} NK cells of MS patients. PBMCs were stimulated with cytokines (IL-2 or IL-12) or left unstimulated without for 24 h. Percentages of IFN-γ-positive CD56^{bright}CD16^{-}, CD56^{dim}CD16^{+} and total NK cells after stimulation with the indicated cytokines were determined. (A) One representative MS patient is shown. (B) Twenty MS patients and 25 healthy donors (HD) are summarized. Asterisks indicate statistical significance (**P = 0.001, *P = 0.017).

frequency of CEM CD4^{+} T cells, primary T-cell blasts and the K562 cell line undergoing spontaneous cell lysis was consistently lower than 10% (Figs 5 and 6). Coculture with non-stimulated and activated effector cells substantially increased target cells lysis. The extent of NK cell-mediated cytotoxicity varied among both patients and controls. While the cytotoxic activity of NK cells tended to be higher in some MS patients for CEM CD4^{+} T cells and primary T-cell blasts and lower for the K562 cell line, the overall difference was not statistically significant for any of the conditions tested (Figs 5 and 6). We additionally determined spontaneous cytotoxicity toward the human erythromyeloblastoid leukemia cell line K562 by sorted NK cells and found no differences between patients and controls (mean ± SEM of TO-PRO+/PKH+ cells in HD versus MS: 55.1 ± 8.1 versus 40.7 ± 6.2; Fig. 6). These data do not argue for an impaired cytolytic potential of NK cells in MS and indicate that NK cell effector functions attributed to distinct NK cell subsets are differentially affected in patients with MS.

Impaired cytokine induced proliferation of NK cells from MS patients

In order to confirm our data of impaired expansion of CD56^{bright}CD16^{-} NK cells, we recruited a second cohort of MS patients and healthy controls from Marburg, Germany (Table 2). Similar to our first patients' cohort, *ex vivo* frequencies of CD56^{bright}CD16^{-} and CD56^{dim}CD16^{+} NK cells were unchanged in comparison with HDs (Fig. 7A). However, after 6 days of IL-12 stimulation *in vitro*, CD56^{bright}CD16^{-} NK cells accumulated less in MS patients than in healthy controls, while CD56^{dim}CD16^{+} remained at similar frequencies (Fig. 7B). In addition, proliferation of purified total NK cells and the NK cell subsets as assessed by CFSE dilution after IL-12 stimulation *in vitro* was compromised, even so the decrease in CD56^{bright}CD16^{-} NK cell proliferation did not reach statistical significance (Fig. 7C). Thus, NK cell proliferation and CD56^{bright}CD16^{-} NK cell accumulation are cell intrinsically deficient in MS patients. These data confirm in an independent MS cohort decreased function of CD56^{bright}CD16^{-} NK cells in MS patients.

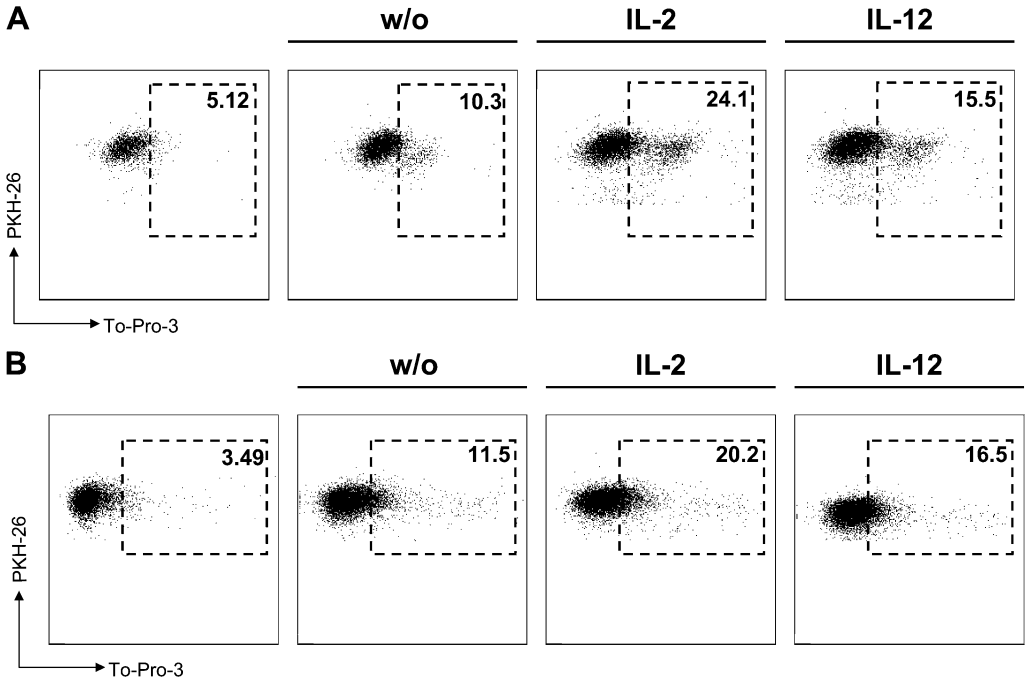

Fig. 4. NK cell cytotoxicity against T cell blasts. NK cells kill the $CD4^+$ T-cell lymphoblast-like cell line CEM, constitutively expressing NKG2D ligands (A) and allogeneic primary $CD4^+$ T cell blasts (B). PBMCs were stimulated with IL-2 or IL-12 for 72 h. Primary $CD4^+$ T cells were stimulated with PHA (2.5 ng ml^{-1}) for 48 h. All experiments were performed at a NK cell:target cell ratio of 10:1. NK cell cytotoxicity was assessed as percentage of TO-PRO-3-positive (membrane integrity deficient) cells among the PKH-26-labeled target cells after 4 h coculture with the indicated NK cell lines. Activated NK cell lines from 1 of 25 healthy blood donors are shown.

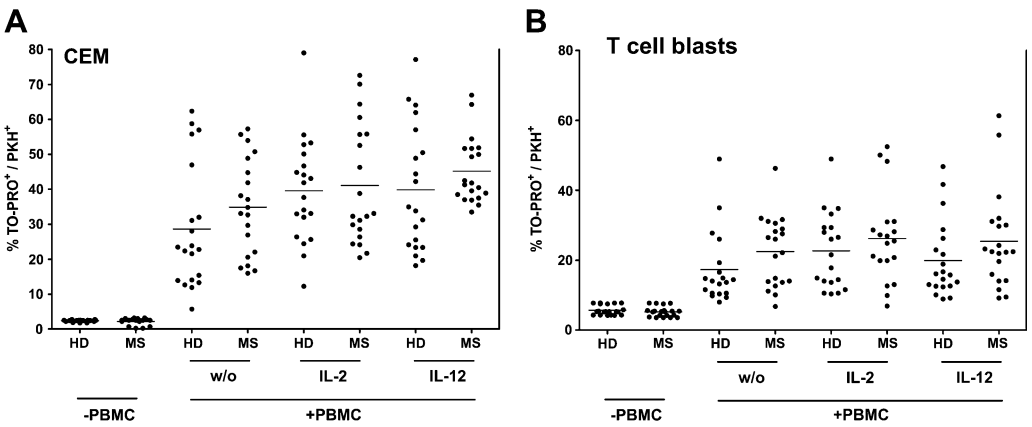

Fig. 5. MS patients do not differ from healthy donors (HDs) in their NK cell cytotoxicity against T cell blasts. Cytotoxic activity of unstimulated without and cytokine (IL-2 or IL-12)-activated NK cells toward (A) a $CD4^+$ T cell lymphoblast-like cell line constitutively expressing NKG2D ligands (CEM) and (B) PHA-activated T cell blasts is unchanged in MS. NK cell cytotoxicity was assessed as percentage of TO-PRO-3-positive (membrane integrity deficient) cells among the PKH-26-labeled target cells after 4 h coculture with the indicated NK cell lines. Twenty MS patients and 25 HDs are summarized.

Discussion

Our study provides evidence for a functional deficiency of $CD56^{bright}CD16^-$ NK cells in untreated patients with MS. *In vitro* activation of NK cells from MS patients, even with limiting concentrations of IL-12, resulted in impaired accumulation of $CD56^{bright}CD16^-$ NK cells. Furthermore, $CD56^{bright}CD16^-$ NK cells from MS patients produced reduced amounts of IFN-γ following IL-12 stimulation, whereas cytolytic NK cell functions toward the classical target cell line K562, allogeneic $CD4^+$ T cells, constitutively expressing NKG2D ligands, and allogeneic primary $CD4^+$ T cell blasts were unchanged in MS. These data suggest a selective impairment of $CD56^{bright}CD16^-$ NK cells and with IFN-γ production as well as proliferation compromised hallmark effector functions of this NK cell subset in MS patients.

Studies in animals models of MS suggested that NK cells limit autoimmune tissue inflammation via killing of encephalitogenic effector T cells since antibody-mediated NK cell depletion exacerbated EAE pathology in different rodent strains associated with enhanced T-cell responses to the

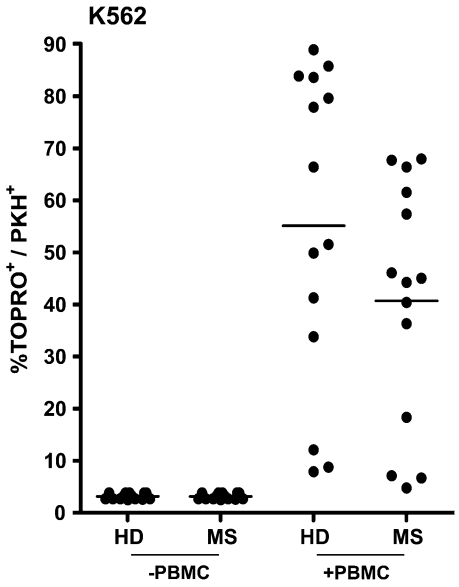

Fig. 6. NK cell cytotoxicity against erythroleukemic cells. Cytotoxic activity of unstimulated sorted NK cells toward the standard target cell line K562 is unchanged in MS. NK cell cytotoxicity was assessed as percentage of TO-PRO-3-positive (membrane integrity deficient) cells among the PKH-26-labeled target cells after 4 h coculture with the indicated NK cell line. Fourteen MS patients and 14 healthy donors (HDs) are summarized.

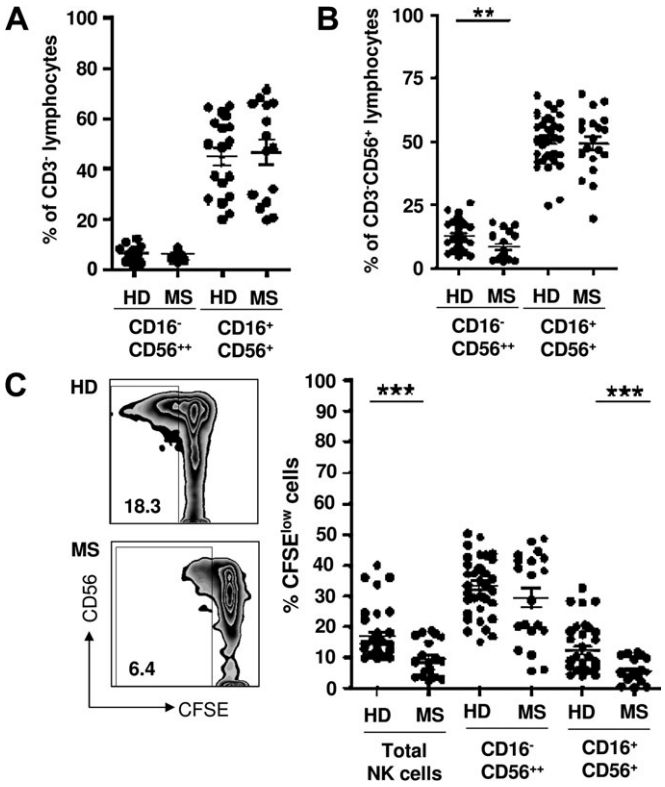

Fig. 7. Accumulation and proliferation of NK cells are impaired in an independent cohort of MS patients and are independent from T cells. The cohort of MS patients from Marburg, Germany (see Table 2), was analyzed in comparison with HDs in the NK cell subset frequencies *ex vivo* (A). After 6 days incubation with IL-12, the accumulation of $CD56^{bright}CD16^{-}$ NK cells was quantified (B). The proliferation of purified total NK cells and the indicated NK cell subsets of MS patients were analyzed in comparison with HDs by CFSE dilution (C). Representative contour blots of one HD and one MS patient are shown. Data of 12 MS patients and 12 HDs measured in triplicates are summarized. Asterisks indicate statistical significance (***P = 0.0004, **P =0.026).

immunizing neuroantigen *in vitro* (26, 30). In addition, innate lymphocytes, via IFN-γ production, are reported to inhibit the development of pathogenic T_h1 and T_h17 effector responses in experimental models of autoimmune diseases (31, 32). Several studies reported that individuals with autoimmune syndromes such as MS and systemic lupus erythematodes have fewer blood NK cells than healthy subjects and NK cells isolated from patients with MS were found not only to be decreased in frequency but also to be impaired in effector function (33–37). On the contrary, other studies found no differences in NK cell frequencies and functions between patients with MS and other autoimmune diseases compared with healthy controls (38–41). These seemingly contradictory findings are difficult to interpret since all the aforementioned studies differed widely in their criteria used to classify NK cells. Some of the older reports did not distinguish between NK cells and NKT cells and none of the above studies differentiated between $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells. Moreover, the assays and protocols used to study NK cell frequencies and functions, as well as the patient populations studied, varied significantly (42). In our study, differences between MS patients and controls were confined to the $CD56^{bright}CD16^{-}$ subset and undetectable unless NK cells were exposed to activating cytokines *in vitro*.

Among the soluble factors, IL-12 is thought to be the most pivotal signal-enhancing factor for NK cell effector functions in humans and in mice (8, 10,43–45). Yet, a recent study suggests that IL-15 acts as an even earlier and more crucial regulator of NK cell differentiation and function, at least in mice (9). After *in vivo* stimulation with TLR ligands or bacterial and viral infection, it was shown that myeloid $CD11c^{high}$ DCs need to prime NK cells via presentation of IL-15 to produce IFN-γ and become cytotoxic against a MHC class I low cell line-expressing NKG2D ligands. In humans, we have recently shown that IL-15Rα accumulates at the synapse between DCs and NK cells and contributes to NK cell survival (22). Although individuals with a history of IM, a MS-predisposing syndrome (24, 25), are reported to lack expression of IL-15Rα on NK cells (23), we could exclude that the poor expansion and cytokine production of $CD56^{bright}CD16^{-}$ NK cells are due to an impaired expression of the high-affinity IL-15 receptor. Instead, persistent NK cell activation, as can be envisioned during relapsing-remitting MS, could exhaust the NK cell compartment, differentiating more $CD56^{bright}CD16^{-}$ NK cells into $CD56^{dim}CD16^{+}$ NK cells. Both IL-12 and IL-15, monokines produced during immune responses as well as the lymphokine IL-2 can differentiate immunoregulatory into cytotoxic NK cells *in vitro* and *in vivo* (7, 46, 47). Consistent with this hypothesis, we found decreased immunoregulatory NK cell functions (accumulation and cytokine production by $CD56^{bright}CD16^{-}$ NK cells),

but normal NK cell cytotoxicity, mainly mediated by $CD56^{dim}CD16^{+}$ NK cells.

It remains to be determined whether the deficiency in $CD56^{bright}CD16^{-}$ NK cell function contributes to the immunopathology of MS or reflects an epiphenomenon without any consequence for disease development and progression. However, the fact that $CD56^{bright}CD16^{-}$ NK cells expand during effective immunotherapies (27, 48, 49) and correlate with the inhibition of contrast-enhancing lesions on brain magnetic resonance imaging (MRI) during immunotherapy with a monoclonal antibody targeting the IL-2R alpha chain, i.e. daclizumab (27), suggests that these innate lymphocytes exert beneficial immunoregulatory functions in MS.

In summary, we have demonstrated that untreated patients with MS show impaired regulatory NK cell responses, which could be attributed to the abundant NK cells in secondary lymphoid tissues, namely $CD56^{bright}CD16^{-}$ NK cells. The functional deficiency in MS is restricted to IFN-γ production upon immune activation and does not affect the cytolytic potential of NK cells. These data reinforce the notion that NK cells, originally named after their ability to mediate spontaneous cytotoxicity toward certain tumor cell lines, regulate autoimmune responses and suggest that $CD56^{bright}CD16^{-}$ NK cells should be further explored for their immunotherapeutic potential in MS.

Supplementary data

Supplementary data are available at *International Immunology* Online.

Funding

Dana Foundation's Neuroimmunology program, the Burroughs Wellcome Fund, the Starr Foundation, the National Cancer Institute (R01CA108609 and R01CA101741), the National Institute of Allergy and Infectious Diseases (RFP-NIH-NIAID-DAIDS-BAA-06-19), the Foundation for the National Institutes of Health (Grand Challenges in Global Health), the Swiss National Science Foundation, the Sassella Foundation and an Institutional Clinical and Translational Science Award (to the Rockefeller University Hospital) to C.M.; Dana Foundation and Irvington Institute's Human Immunology Fellowship, the National Multiple Sclerosis Society, the Swiss National Science Foundation, the Swiss Multiple Sclerosis Foundation, the Betty and David Koetser Foundation, the Ernst Schering Foundation, the Baxter Research Grant Program and the Gemeinnützige Hertiestifung to J.D.L.; Marie-Heim-Vögtlin Programme of the Swiss National Foundation to A.L.

Acknowledgement

The authors would like to thank Florian Seitz for his help in collecting the MS patients samples in Marburg, Germany.

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