Splenic natural killer cell activity in two models of experimental neurodegenerative diseases

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Abstract

Natural killer (NK) cells are antitumour/anti-viral effectors and play important roles in shaping the immune system, but their role in neurodegenerative diseases is not clear. Here, we investigated the fate of these cells in two neurodegenerative diseases. In the first model, the activity of NK cells was examined in mice with experimental autoimmune encephalomyelitis (EAE) treated with glatiramer acetate (GA or Copaxone), a drug used to treat EAE in animals and multiple sclerosis in human. The second disease model is twitcher (*GalC^{twi}/GalC^{twi}*) mice, which represents an authentic model of human Krabbe's disease. Administration of GA ameliorated EAE in SJL mice corroborated with isolating NK cells that expressed higher killing than cells isolated from vehicle-dosed animals against immature or mature dendritic cells (DCs). However, this drug showed no effect on the numbers of NK cells or the expression of CD69 molecule. On the other hand, NK cells either disappeared from the spleens or were present in low numbers in the white pulp areas of *GalC^{twi}/GalC^{twi}* mice, which have increased D-galactosyl- β 1-1'-sphingosine (GalSph) levels. Analysis by confocal microscopy shows that NK cells found in the spleens of *GalC^{twi}/GalC^{twi}* mice were apoptotic. Incubating NK cells *in vitro* with GalSph induced the apoptosis in these cells, confirming the results of twitcher mice. Our results provide the first evidence showing that amelioration of EAE in mice is corroborated with NK cell lysis of antigen-presenting DCs, whereas NK cell distribution into the spleen is altered in a devastating lipid disorder corroborated with induction of their apoptosis.

Keywords: NK cells • dendritic cells • EAE • globoid cell leukodystrophy • glycosphingolipids • glatiramer acetate

Introduction

The immune system is composed of a network of cells and proteins that act in concert to defend the body against diseases. There is increasing evidence that key components of the innate immune system such as natural killer (NK) cells are instrumental in disease control or progression. Although NK cells were first discovered as antitumour effector cells (reviewed in reference 1), these cells perform several important functions such as defending against viral infections [2]. Treatment of AIDS patients with the drug AZT results in a rebound of NK cell activity and number, associated

with improved prognosis [3, 4], indicating that NK cells are important for killing HIV-infected cells. Other virus infections are also resolved by NK cells [5]. In the murine system, infection with murine cytomegalovirus (MCMV) leads to the accumulation of NK cells in the liver and spleen of infected mice [6, 7]. Collectively, these results suggest that NK cells are effectors that eradicate viral infections.

NK cells have a certain pattern of tissue localization. The majority of human NK cells are present in the blood circulation but are recruited to sites of inflammation by chemokines and lysophospholipids [8, 9]. On the other hand, mouse NK cells are predominantly accumulated in the splenic red pulps (RPs) and marginal zones (MZs), but are rarely present in the splenic white pulps (WPs). In areas of intense immune activity such as inflammatory sites, cognate interactions are a common feature of antigen-specific immune response, where intimate cell contacts govern critical events such as antigen presentation and delivery of T cell help to cytotoxic T lymphocytes and B cells.

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With respect to the interaction among dendritic cells (DCs) and NK cells, a number of studies highlighted the importance of such interaction in the regulation of DC maturation as well as NK cell activation. The interaction between NK cells and DCs is bidirectional and involves cell-to-cell contact. DCs activate NK cells by enhancing their proliferation, cytotoxic activity and interferon (IFN)- γ production [10]. In return, activated NK cells provide either maturation signals for DCs or induce their death by direct killing [11]. Dendritic cells are specialized antigen-presenting cells that present antigens and, consequently, activate T cells. Myeloid human immature dendritic cells (iDCs) are generated from monocytes exposed to pro-inflammatory cytokines such as granulocyte macrophage-colony-stimulating factor (GM-CSF) in the presence of interleukin (IL)-4 or IFN- α [12]. These immature DCs have certain phenotypes and are capable of capturing antigens, but have little ability to present them because of their low expression of co-stimulatory molecules such as CD80, CD83 and CD86. Bacterial or viral infections promote the maturation of these cells to become mature dendritic cells (mDCs), which up-regulate the expression of co-stimulatory molecules and, hence, become potent antigen-presenting cells that promote T-cell activation [13].

The role of NK cells in neurodegenerative diseases has not been extensively studied. There are conflicting reports about the role of these cells in experimental autoimmune encephalomyelitis (EAE). It has been reported that NK cells either ameliorate the disease [14, 15] or exacerbate it [16, 17]. Glatiramer acetate (GA), a drug used to treat EAE in animals and multiple sclerosis (MS) in human [18], enhances human NK cell lysis of DCs when incubated *in vitro* with NK cells [19]. However, the effect of GA on NK cells in the setting of neurodegenerative diseases is not known. Here, we have investigated the effect of GA on NK cell activity in EAE mice treated with this drug.

Further, we examined the status of NK cells in twitcher (*Galc^{twi}/Galc^{twi}*) mice, which is a mouse model of globoid cell leukodystrophy (GLD) or Krabbe's disease. The pathology of GLD is characterized by the destruction of oligodendrocytes, reduced myelin formation and accumulation of globoid cells, not only in humans but also in canines, domestic cats and rhesus monkeys [20–22]. Accumulation of the toxic lipid D-galactosyl- β 1–1' sphingosine (GalSph) in the brain is thought to cause the disease [23, 24]. GalSph is virtually absent from normal brain or other tissues [25], but accumulates in the brain of Krabbe's patients due to the deficiency of the enzyme galactosyl ceramidase (GALC). In *Galc^{twi}/Galc^{twi}* mice, which have mutations in their *GALC* gene and represent an authentic model of human Krabbe's disease [26], GalSph is increased in the kidneys, liver, spleen and brain of these animals. For example, the level of this lipid can reach up to 800 pmol/mg protein in the brain of twitcher mice [27], and about 60 ng/100 mg wet tissue weight in the spleen of these mice [25]. This is corroborated with increased infection and low immune response in these animals [28]. Hence, we reasoned that the latter events might be related to lower NK cell activity in these mice. Therefore, we examined the status of NK cells and herein report that these cells are damaged in the spleens of *Galc^{twi}/Galc^{twi}* mice.

Materials and methods

Reagents

Proteolipid peptide (PLP) was purchased from Multiple Peptide Systems (San Diego, CA, US). Pertussis toxin (PTX) was obtained as a salt-free, lyophilized powder from Calbiochem (San Diego, CA, USA, or Oslo, Norway). PBS used for the study was sterile and endotoxin-free. Incomplete Freund's adjuvant and desiccated (killed and dried) *Mycobacterium tuberculosis* (MTB) were purchased in powdered form from Difco (San Diego, CA, USA).

Animals

Wild-type (WT) male C57BL/6J and twitcher (*Galc^{twi}/Galc^{twi}*) C57BL/6J mice were purchased from Charles River (Wilmington, MA, USA). For EAE induction, female SJL mice were purchased from Taconic Farms, Inc. (Germantown, NY, USA, or Ejby, Denmark). Mice used in this study were between 30 and 35 days of age and were kept under a veterinary supervision in the animal facility of the University of Oslo (Oslo, Norway) or BioQuant, Inc. (San Diego, CA, USA).

EAE induction

On day 0, female SJL mice were immunized with 200 μ g of PLP_{139–151} peptide emulsified in complete Freund's adjuvant (CFA) in the right and left flanks. Following each injection, 100 ng of PTX was injected *via* the tail vein route. EAE clinical score was measured according to the following scoring scheme. 0 = no clinical disease, 1 = tail flaccidity, 2 = hind limb weakness, 3 = hind limb paralysis, 4 = forelimb paralysis and 5 = moribund or death.

Treatment of mice with GA and isolation of NK cells and DCs

SJL mice were divided into two groups. The first group was dosed with PBS as a vehicle, and the second was injected subcutaneously (SC) with 50 μ g/mouse GA (Teva Neuroscience, Kansas City, MO, USA) on days 0, 6, 14 and 21. On day 7, bone marrow (BM) cells were collected from the tibia and femur of three mice to generate DCs. The BM cells were incubated at 5×10^6 cells/ml in 100-mm dishes with 25 ng/ml recombinant mouse GM-CSF and 6 ng/ml recombinant mouse IL-4 (both from Pepro Tech Ltd., London, England). Four days later, half of the culture supernatants were removed and the cultures were replenished with fresh media containing 25 ng/ml GM-CSF and 6 ng/ml IL-4. Three days after this, the supernatants were removed, centrifuged and added back to the cultures without the pellets. Cells in this preparation are considered as iDCs, as previously described [29]. To two of the plates, 1 μ g/ml lipopolysaccharide (LPS) was added to generate mDCs [29]. These cells were collected on day 9 and used as targets for NK cells.

Two methods were used to generate GA-primed NK cells. In the first method, splenocytes were isolated from mice dosed on days 0 and 6 with 50 μ g/mouse GA. These cells were adhered to 100-mm petri dishes to remove adherent cells such as monocytes. Two hours later, the cells were collected and incubated (2×10^6 cells/ml) for 2 days with 200 U/ml IL-2.

After this, the non-adherent cells were removed, and the conditioned media was added back to the flasks. The cells were allowed to grow for additional 7 days in culture (total incubation time with IL-2 was 9 days). These cells were considered IL-2-activated NK (IANK) cells, as previously described [30], and were used to lyse iDCs and mDCs. In the second method, SJL mice were injected SC with 50 μ g/mouse GA or PBS as a vehicle for a total of 16 days, coincided with three rounds of GA or vehicle dosing (days 0, 6 and 14). From both GA-treated and vehicle-treated mice, nylon wool column (NWC)-non-adherent cells were collected and examined for their ability to lyse iDCs and mDCs.

Flow cytometric analysis

Splenocytes were layered over a histopaque gradient and mononuclear cells were collected and separated by NWC, a procedure where B cells and monocytes adhere to the nylon wool, whereas T cells and NK cells are non-adherent. NWC-non-adherent cells were collected and labelled with 1 μ g/ml R-PE-conjugated monoclonal antibody to NK1.1 (Southern Biotech, Birmingham, AL, USA), a cell surface receptor specific for NK cells. Surface labelling of mouse cells with PE-conjugated anti-CD69 (ImmunoTools) was done by incubating 200,000 cells/well in V-bottom 96-well plates with 5 μ g/ml of the antibody in PBS plus Ca^{2+} and Mg^{2+} , 2% foetal calf serum (FCS) and 0.1% sodium azide.

NK cell cytotoxicity assay

Mouse iDCs or mDCs were used as targets. The target cells were incubated at 1×10^6 cells/ml with 5 μ g/ml calcein-AM (Teflabs, Austin, TX, USA) for 1 hr at 37°C. After this, the target cells (10,000/well) and effector cells were plated onto 96-well plates at the indicated E:T cell ratios in triplicate. The plates were spun down at 500 rpm for 5 min. and incubated for 4 hrs at 37°C. After incubation, the plates were centrifuged, supernatants removed and then replaced with PBS. Fluorescence units (FU) were measured in Cytofluor (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) plate reader using 485/528 nm fluorescence filters. Viability was calculated according to the following formula: % viability = FU of targets incubated with NK cells (experimental) minus FU of targets incubated with Triton-X, divided by FU of targets incubated in media only (total viability) minus FU of targets incubated with Triton X (total lysis). Percent cytotoxicity was then calculated as $100 - \% \text{ viability}$ [31].

Detection of NK and NKT cells in the spleen of mice

The mice were anaesthetized and fixed by transcardiac perfusion at a flow rate of about 7 ml/min (60 ml/mouse) with 4% formaldehyde and 0.05% glutaraldehyde in 0.1 M NaPi (sodium phosphate buffer). The brains and spleens were removed from these mice, immersed in the same fixative for 2 days at 4°C and stored in cold fixative diluted 10 times with buffer. Free-floating Vibratome (Vibratome 1000 plus, Ted Pella, Inc., Redding, CA, USA) sections (50- μ m thick) were treated with 1 M ethanolamine-HCl (pH 7.4) and blocked with 10% FCS in 0.1 M Tris-HCl containing 0.3 M NaCl. Spleen sections were labelled with 5 μ g of R-PE-conjugated anti-NK1.1 (Southern Biotech) for 1 hr at 4°C in the dark with continuous mixing at a low speed. The sections were washed extensively with PBS, dried and labelled with 5 μ g of FITC-conjugated hamster anti-mouse CD3 (Southern Biotech) for 45 min. at 4°C in the dark. As a

control, spleen sections were stained with PE-conjugated IgG and FITC-conjugated IgG (ImmunoTools). The sections were examined using confocal microscopy (LSM 5 Pascal).

Preparation of NK cells from C57BL/6J mice and induction of apoptosis

The spleen cells were harvested from WT C57BL/6J mice. NK cells were purified using EasySep mouse NK cell enrichment kit, as suggested by the manufacturer (StemCell Technologies Europe, Grenoble, France). NK cells (1×10^6 /ml) were either left intact or were incubated with buffer or with 50 or 100 μ M GalSph (Avanti Polar Lipids, Alabaster, AL, USA) for 24 hrs at 37°C in fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich, Oslo, Norway). The cells were extensively washed and then examined for apoptosis using two different methods. In the first method, the cells were incubated with 5 μ g of rhodamine-phycoerythrin (R-PE)-conjugated anti-NK1.1 for 1 hr at 4°C in the dark and then labelled with 5 μ g of fluorescein isothiocyanate (FITC)-conjugated recombinant annexin V (ImmunoTools, Friesoythe, Germany) for 1 hr at 4°C. They were extensively washed and then examined using confocal microscopy (LSM 5 Pascal; Carl Zeiss, Heidelberg, Germany). In the second method, a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay utilizing the APO-BRDU kit, (Becton-Dickinson Pharmingen, San Jose, CA, USA), a two-colour staining method for labelling DNA breaks and total cellular DNA to detect apoptotic cells, was used. Apoptotic cells were detected by flow cytometry.

Detection of apoptotic cells in the spleens of twitcher mice

Wild-type as well as *GalC^{twi}/GalC^{twi}* mice were injected intravenously with 1 mg of FITC-conjugated recombinant annexin V (ImmunoTools). One hour later, the spleens were isolated and sections were prepared and stained with 5 μ g of R-PE-conjugated anti-NK1.1 for 1 hr at 4°C in the dark. They were extensively washed and then mounted on a Fluoromount G (Electron Microscopy Sciences, Hatfield, PA, USA). The sections were examined using confocal microscopy (LSM 5 Pascal).

Electron microscopy procedures

Sections of the brain from both WT C57BL/6J and twitcher (*GalC^{twi}/GalC^{twi}*) C57BL/6J mice were incubated with 1% OsO₄ for 1 hr in 0.1 M NaPi, dehydrated in graded ethanol and propylene oxide and embedded in Durcupan ACM (Sigma-Aldrich). Ultrathin sections (<100 nm in thickness) were cut from the embedded material. These sections were lightly contrasted with 10 mg/ml uranyl acetate for 10 min. and 3 mg/ml lead citrate for 70 sec., properly washed with pure water and then examined using a Philips CM10 electron microscope (Philips Electronics Inc., Mahwah, NJ, USA).

Statistics

Significant values were generated using the Student t-test. Area under curve (AUC) was calculated by Prism program (Graphpad Prism, San Diego, CA, USA).

Fig. 1 GA administration ameliorates EAE in SJL mice. **(A)** Mice with EAE were either dosed with vehicle (PBS) or dosed with GA on days 0, 6, 14 and 21. Mean clinical score for 12 mice in each group is shown (of note, one of the mice in the vehicle-treated group died). **(B)** Area under curve (AUC) of EAE clinical score measured over 45 days. Each dot represents the AUC generated from one mouse during the entire period of measurement, that is, 45 days.

Results

To study the effect of GA on EAE clinical score, the mice were divided into two groups. The first group was dosed with vehicle (PBS), and the second group was injected with 50 μ g/mouse GA (Copaxone) on days 0, 6, 14 and 21 after the initiation of the disease. The results demonstrate that GA inhibited EAE clinical score between 10 and 20 days after dosing, with maximal effect after 16–17 days (Fig. 1A). However, this effect disappeared between 20 and 28 days and resurged after 28 days. AUC measurements done over the entire period of dosing (45 days) show that GA significantly inhibited the clinical EAE score ($P < 0.05$ as compared with the control; Fig. 1B).

To correlate these findings with the involvement of NK cells and DCs in EAE, iDCs and mDCs were generated from mice that were dosed twice on days 0 and 6 with 50 μ g/mouse GA or with PBS as a vehicle control. On the other hand, NK cells were generated by two different methods. In the first method, cells were isolated from the spleens of mice that were dosed twice on days 0 and 6 with either 50 μ g/mouse GA or with vehicle and then incubated *in vitro* with IL-2 for 9 days. The other preparations include purifying NWC-non-adherent cells after 16 days from the spleens of mice that were dosed three times on days 0, 6 and 14 with 50 μ g/mouse GA or with vehicle. Both freshly isolated and IANK cells were examined for their ability to lyse iDCs or mDCs isolated from vehicle-dosed or GA-dosed mice. Although several E:T cell ratios were used, for simplicity, only the results of 50:1 and 10:1 E:T cell ratios of freshly isolated NK and IANK cells, respectively,

are shown. Exposing the mice to GA enhanced NK cell lysis against DCs when compared with the lysis of NK cells isolated from vehicle-dosed animals. Both freshly isolated NK cells and those that were cultured *in vitro* with IL-2 for 9 days isolated from GA-dosed mice showed significantly higher killing than NK cells collected from vehicle-dosed mice against iDCs isolated from vehicle-dosed mice ($P = 0.05$ and 0.03 , respectively; Fig. 2A). NK and IANK cells collected from GA-dosed animals expressed significantly higher killing than NK and IANK cells collected from vehicle-dosed animals against iDCs isolated from GA-dosed animals ($P = 0.04$ and 0.03 , respectively; Fig. 2B). Similar findings were observed when NK or IANK cells collected from GA-dosed animals were examined for their ability to lyse mDCs isolated from vehicle-dosed mice ($P = 0.03$ and 0.05 for NK and IANK cells, respectively, as compared with the lysis of NK or IANK cells collected from vehicle-dosed mice; Fig. 2C). The lysis of mDCs isolated from GA-dosed mice followed similar pattern; that is, NK or IANK cells isolated from GA-dosed mice showed higher killing than NK or IANK cells collected from vehicle-dosed mice ($P = 0.05$ and 0.02 , respectively; Fig. 2D).

To demonstrate whether GA affects the viability of NK cells, we determined the numbers of NK cells in the spleens. The results show that there were no differences in the expression (Fig. 3A *versus* Fig. 3B) or the percentages of NK1.1⁺ cells (Fig. 3E) among GA-treated and vehicle-treated animals. Similarly, there was no effect of GA dosing on the expression (Fig. 3C *versus* Fig. 3D) or the percentages of splenocytes expressing the early activation marker CD69 (Fig. 2F).

Fig. 2 GA enhances NK cell lysis of immature dendritic cells (iDCs) and mature dendritic cells (mDCs). **(A)** NK cells were isolated on day 16 from mice either dosed with vehicle or with 50 μ g/mouse GA on days 0, 6 and 14. In addition, NK cells were collected from the spleens of vehicle-dosed or GA-dosed mice on days 0 and 6. The latter cells were cultured *in vitro* with IL-2 for 9 days and were designated as IL-2-activated NK (IANK) cells. All four cell types were examined for their ability to lyse immature dendritic cells (iDCs) cells isolated from vehicle-dosed animals. E:T cell ratios used were 50:1 for freshly isolated NK cells and 10:1 for IANK cells. **(B)** This is similar to **(A)**, except that iDCs were generated from GA-dosed animals. **(C)** Bone marrow cells were isolated on day 7 from animals dosed with vehicle on days 0 and 6. These cells were cultured *in vitro* with GM-CSF plus IL-4 for 7 days and then incubated for additional 2 days with LPS, as described in the Materials and methods section. These mature dendritic cells (mDCs) were used as targets for the four different subsets of NK cells. **(D)** Similar to **(C)**, except that mDCs isolated from GA-dosed animals were used as targets. All results are mean $\pm$ SEM of three separate experiments. In each experiment, three mice were used to isolate DCs and two or three mice for NK cells.

Whether NK cells might play a role in other neurodegenerative diseases was next examined, taking advantage of the availability of a mouse strain that resembles GLD disease in human. Surprisingly, all attempts to isolate NK cells from the spleens of these mice failed. Hence, we recovered only minimal numbers of NK cells from the spleens of *GalC^{twi}/GalC^{twi}* mice, which were not enough to perform any biological assay. Consequently, we performed electron microscopy and immunohistochemistry studies to understand the reasons for the disappearance of NK cells from the spleens of these mice. In the central nervous system (CNS) of WT or twitcher mice, we did not observe any accumulation of NK1.1⁺ cells (data not shown). In the brain, the axons of *GalC^{twi}/GalC^{twi}* mice were thinner and were mildly myelinated. Also, the myelin sheath was irregular and fuzzy (Fig. 4C), when compared with the axons of WT animals (Fig. 4A). Moreover, edema accumulation was noticed inside the myelin sheath of the axons in the white matter of *GalC^{twi}/GalC^{twi}* mice (E in Fig. 4C). The synaptic vesicles in the parallel fibres (PF) of twitcher mice were more condensed as compared with WT animals (Fig. 4D *versus* Fig. 4B). The significance of the latter finding is not clear. Collectively, these findings indicate that the axons as well as other parts in the brain of twitcher mice are damaged.

Immunohistological examination of spleen sections revealed that NK cells were present in the RP and MZ areas of WT animals, but these cells either disappeared or were present in low numbers in the WP areas of splenic *GalC^{twi}/GalC^{twi}* mice (data not shown). Whether the accumulation of cells inside the spleen is due to the infiltration of NK or the rare NKT cells was next investigated. For this, spleen sections were labelled with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3. Sections labelled with control antibodies showed only sporadic staining (Fig. 5A). Several cells labelled with PE-conjugated anti-NK1.1 were observed in the RP areas of the spleens of WT animals (Fig. 5B, white rectangles). Co-localization showed a combination of red cells labelled with PE-conjugated anti-NK1.1 (NK cells) and yellow/orange cells labelled with both PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 (*i.e.* NKT cells) in the RP areas of WT animals (Fig. 5C, white rectangles). However, the majority of NK cells did not stain with anti-CD3, suggesting that NK cells are more predominant in the spleen of mice than the rare NKT cells. On the other hand, few NK cells were captured in the WPs of *GalC^{twi}/GalC^{twi}* animals (Fig. 5D), suggesting that the accumulation of NK cells is altered in the spleens of these mice.

Although low numbers of NK cells were seen in the spleens of *GalC^{twi}/GalC^{twi}* mice, these were difficult to recover and examine

Fig. 3 GA does not modulate the numbers of NK cells or the expression of CD69 molecule. (A) and (B) Representative histograms showing the expression of NK1.1⁺ cells in the spleen of vehicle- or GA-treated mice, respectively. Background staining with negative (cells only) or isotype controls (anti-IgG) is also shown. (C) and (D) Representative histograms showing the expression of CD69⁺ cells in the spleen of vehicle- or GA-treated mice, respectively. Background staining with negative or isotype controls is also shown. (E) Results of flow cytometric analysis showing the percentages of NK cells isolated from mice treated with either vehicle or GA. Mean $\pm$ SEM of four different experiments are shown. (F) Results of flow cytometric analysis showing the percentages of CD69⁺ cells isolated from mice treated with either vehicle or GA. Mean $\pm$ SEM of four different experiments are shown.

Fig. 4 Abnormalities in the brains of twitcher mice. (A) and (B) The brains of wild-type C57BL/6J mice. (C) and (D) The brains of *Galc^{twi}/Galc^{twi}* C57BL/6J mice. All sections were examined by electron microscopy. Double-edge white arrow in (C) shows edema around the axons but inside the myelin sheath (designated as E). Note the difference in the thickness of the axons and the myelin sheath between (A) and (C). The synaptic vesicles in the parallel fibres of twitcher mice cerebellum are more pronounced than those in wild-type counterparts. AX, axon; M, mitochondria; PF, parallel fibres. Scale bars are 1 μ m.

Fig. 5 Confocal microscopy examination of the presence of NK and NKT cells in the spleens of wild-type and twitcher mice. **(A)** The binding of control FITC-conjugated and PE-conjugated antibodies. **(B)** Spleens of wild-type mice showing NK cells labelled with R-PE-conjugated anti-NK1.1 (white rectangles). **(C)** Co-localization, where NK cells (red) and NKT cells (yellow) accumulate in the RP of the spleens of wild-type mice (white rectangles). **(D)** The accumulation of R-PE-labelled NK1.1⁺ cells inside the WP of *Galc^{twi}/Galc^{twi}* mice (arrows). Scale bars are 100 μ m.

Fig. 6 NK cells in the spleens of *Galc^{twi}/Galc^{twi}* mice are apoptotic. **(A)** Spleen sections from wild-type animals injected with FITC-conjugated annexin V were stained *in vitro* with R-PE-conjugated anti-NK1.1. White arrows show cells stained with PE-conjugated anti-NK1.1. **(B)** Similar to **(A)** except that the spleens were isolated from *Galc^{twi}/Galc^{twi}* mice. NK cells labelled with PE-conjugated anti-NK1.1 were also labelled with FITC-conjugated annexin V and are seen as yellow/orange cells. Scale bars are 100 μ m.

in vitro. Because we previously reported that GalSph induces the *in vitro* apoptosis of human NK cells [32], and to demonstrate that NK cells in twitcher mice are also destroyed by apoptosis, spleen sections were prepared from WT and *Galc^{twi}/Galc^{twi}* mice that were injected with FITC-conjugated recombinant annexin V. These sections were stained with R-PE-conjugated anti-NK1.1. Under these conditions, NK1.1⁺ cells were observed in the RP area of WT animals (Fig. 6A). Very few cells in the spleens of WT animals were stained with FITC-conjugated annexin V, indicating that there is no significant degree of apoptosis taking place in the spleens of WT animals (Fig. 6A). In *Galc^{twi}/Galc^{twi}* mice, where some NK cells accumulate in the RP or WP areas, most of these cells were labelled with both dyes (Fig. 6B). In fact, the majority of these cells agglutinated to each other, suggesting that NK cells whether in the RP or WP of twitcher mice are dying.

To ascertain that the apoptosis seen *in vivo* may be related to the effect of GalSph, we incubated purified NK cells isolated from

WT C57BL/6J mice with 50 μ M of this lipid. NK cells incubated with vehicle were used as control. After 24 hrs, the cells were washed and incubated with FITC-conjugated annexin V and R-PE-conjugated anti-NK1.1. The results demonstrate that NK cells incubated overnight with vehicle and then stained with FITC-conjugated annexin V (Fig. 7A) and R-PE anti-NK1.1 (Fig. 7B) did not merge, with only few cells labelled with both dyes (Fig. 7C), suggesting that the majority of NK cells are not apoptotic when they were incubated overnight with vehicle only. In contrast, most NK cells incubated with GalSph and then labelled with FITC-conjugated annexin V (Fig. 7D) and R-PE anti-NK1.1 (Fig. 7E) were apoptotic as there was a merge among cells labelled with both dyes (Fig. 7F). Of note, shorter incubation time with the lipid (e.g. 2 hrs) did not induce apoptosis (data not shown).

Further, we used the TUNEL assay to detect damaged DNA after incubating NK cells with GalSph. In this method, brominated deoxyuridine triphosphate (BrdU) is incorporated into the

Fig. 7 D-galactosyl-β1-1' sphingosine (GalSph) induces *in vitro* apoptosis of mouse NK cells as determined by labelling with FITC-conjugated annexin V. (A) NK cells incubated overnight with vehicle were stained with 5 μM FITC-conjugated annexin V. (B) NK cells incubated overnight with vehicle were stained with 5 μg R-PE anti-NK 1.1 (C) Merge of (A) and (B). (D) NK cells incubated overnight with 50 μM GalSph were stained with 5 μM FITC-conjugated annexin V. (E) NK cells incubated overnight with 50 μM GalSph were stained with 5 μg R-PE anti-NK 1.1. (F) Merge of (D) and (E).

3'-hydroxylated ends of double-stranded and single-stranded DNA. DNA break sites are identified by labelling with FITC-conjugated anti-BrdU antibody. To this end, NK cells (1×10^6 /ml) were incubated with vehicle, 50 μM or 100 μM GalSph for 24 hrs. After this incubation period, the cells were prepared according to the instructions supplied by the TUNEL assay kit. Results shown in Fig. 8 indicate that, in two different samples, apoptosis was observed in 12.6% and 13.7% of cells incubated with vehicle only (Fig. 8A and 8D, respectively). In contrast, 50 μM GalSph induced apoptosis in 53.1% and 48.3% NK cells (Fig. 8B and 8E, respectively). Even higher percentages of apoptotic cells were observed when NK cells were incubated overnight with 100 μM GalSph, that is, 83.0% and 75.2% (Fig. 8C and 8F, respectively).

Discussion

Although it was clearly established that NK cells are potent effector cells responsible for 'immunosurveillance', that is, detecting

and destroying abnormally developed cells or virally infected cells, their role in neurodegenerative diseases is not clear. Here, we show that administration of GA, a drug used for ameliorating MS/EAE disease, enhances NK cell lysis of DCs. However, GA treatment does not affect NK cell numbers, indicating that the effect of GA is directed towards the activation of these cells and not towards their proliferation. In addition, GA did not affect the expression of CD69 activation marker. These results suggest that GA has no effect on early activation of these cells, in contrast to other drugs that down-regulate the expression of CD69 molecule [33].

Culturing mouse NK cells that were exposed twice to GA *in vivo*, with IL-2 for 9 days *in vitro*, results in NK cells that have high killing of iDCs and mDCs that are isolated from GA-dosed or vehicle-dosed animals. These results suggest two important points: (1) exposure to IL-2, which is also used for therapeutic purposes, does not interfere with the effect of GA, and (2) culturing the cells *in vitro* for a long time (9 days) in the absence of GA does not modulate the effect of GA inherited after exposure to this drug in the animals. The present results clearly indicate that one of the possible mechanisms of GA inhibition of EAE clinical

Fig. 8 D-galactosyl- β 1-1' sphingosine (GalSph) induces *in vitro* apoptosis of mouse NK cells, as determined by the TUNEL assay. (A) and (D) NK cells (1×10^6 /ml) were incubated overnight with vehicle only. (B) and (E) NK cells (1×10^6 /ml) were incubated with 50 μ M GalSph for 24 hrs. (C) and (F) 100 μ M GalSph was incubated with 1×10^6 /ml NK cells for 24 hrs. The cells were then fixed and stained according to the APO-BrdU TUNEL assay procedure. Damaged DNA, which incorporates BrdU, was detected by flow cytometry. Percentages of apoptotic cells are also shown.

disease may be due to its ability to activate NK cells to lyse DCs. Hence, NK cells exposed to GA kill both iDCs and mDCs, which may lead to the inability of the latter to present antigens to autoreactive T cells. Consequently, Th1 stimulation by DCs is detected and controlled by NK cells, confirming the role of these cells as important sentinel cells responsible for the 'immunosurveillance' mechanism as well as regulators of the immune system.

The next question we asked is whether NK cells might play any role in other neurodegenerative diseases, taking advantage of the presence of a mouse model of GLD disease, that is, twitcher mice. This approach is similar to the above approach, where we took advantage of using a mouse model for MS. Patients with storage diseases that involve the nervous system succumb to infections, which are the major cause of death in these patients [34]. The reason for increased rate of infections is not clear. Here, we observed that the distribution of NK cells in the spleens of *GalC^{twi}/GalC^{twi}* mice is altered; that is, NK cells distribute into some splenic WPs of *GalC^{twi}/GalC^{twi}* mice. This pattern of distribution has not been seen before. In contrast, splenic NK cells of normal mice accumulate in the RP and MZ but not in the WP areas.

The reason for the accumulation of NK cells in the WPs of *GalC^{twi}/GalC^{twi}* mice is not yet clear. Because GalSph is increased in the spleens of these mice, our findings continue to support the

view that this lipid accumulates in the WP area and consequently recruits NK cells towards these sites, similar to its effect on human NK cells [32]. The accumulation of NK cells in these areas is corroborated with the induction of their destruction. In fact, it was highly difficult to isolate NK cells from the spleens of *GalC^{twi}/GalC^{twi}* mice to examine their *in vitro* activities, which could be due to their destruction, most probably due to apoptosis as a result of the presence of GalSph. This phenomenon is supported by two findings: (1) NK cells isolated from the spleens of *GalC^{twi}/GalC^{twi}* mice show increased apoptosis and agglutination, indicating that they are dead cells, and (2) culturing mouse NK cells overnight with GalSph induces their apoptosis, as determined by two different methods: the first utilized FITC-conjugated annexin V, and the second was the TUNEL assay. These results support those showing that incubation of human NK cells with GalSph results in inducing apoptosis in these cells [32]. Hence, our results may provide an explanation to the findings that *GalC^{twi}/GalC^{twi}* mice have low immunological activity and can easily accept grafts [28, 35], which could be a result of damaging cells of the innate immune system.

Transplantation of haematopoietic cells from normal mice into syngeneic twitcher mice resulted in increased survival [35, 36]. In human beings, Krivit *et al.* [37] reported a highly successful rate

of recovery in five patients with late-onset GLD after haematopoietic stem cell transplantation. Whether NK cells were present in this mixture of haematopoietic cells has not been examined. One assumes that if NK cells were included, then they may have helped in resolving the infections associated with the disease.

In conclusions, our results show for the first time the involvement of NK cells in two neurodegenerative diseases, that is, EAE and GLD. In the first disease, NK cells proved to be highly important in killing DCs known to present antigens to autoreactive T cells. Whether NK cells lyse only those cells that present antigens to autoreactive Th1 cells, that is, monocyte-derived DCs, as described in this paper, or they may also affect DCs that activate Th2 cells, that is, lymphoid-derived DCs, remains to be investigated. Hence, NK cells are important effector cells that may ameliorate EAE and perhaps MS due to this distinguishing role they play. In the second disease model, NK cells present in the spleens of *GalC^{twi}/GalC^{twi}* mice are damaged due to their apoptosis. Taken together, it is clear that the presence of NK cells is highly impor-

tant for these two neurodegenerative diseases. In autoimmune EAE, NK cells protect the animals by lysing monocyte-derived DCs after treatment with GA, and in the other case, a devastating disease (GLD) is maintained corroborated with the disappearance and destruction of NK cells. On the basis of these findings, it is important that the role of NK cells in other neurodegenerative diseases should be evaluated. If proven, then the next step is to reconstitute these animals with NK cells as therapeutic tools.

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