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Cytotoxic NKG2C⁺ CD4 T Cells Target Oligodendrocytes in Multiple Sclerosis

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The mechanisms whereby immune cells infiltrating the CNS in multiple sclerosis patients contribute to tissue injury remain to be defined. CD4 T cells are key players of this inflammatory response. Myelin-specific CD4 T cells expressing CD56, a surrogate marker of NK cells, were shown to be cytotoxic to human oligodendrocytes. Our aim was to identify NK-associated molecules expressed by human CD4 T cells that confer this oligodendrocyte-directed cytotoxicity. We observed that myelin-reactive CD4 T cell lines, as well as short-term PHA-activated CD4 T cells, can express NKG2C, the activating receptor interacting with HLA-E, a nonclassical MHC class I molecule. These cells coexpress CD56 and NKG2D, have elevated levels of cytotoxic molecules FasL, granzyme B, and perforin compared with their NKG2C-negative counterparts, and mediate significant in vitro cytotoxicity toward human oligodendrocytes, which upregulated HLA-E upon inflammatory cytokine treatment. A significantly elevated proportion of ex vivo peripheral blood CD4 T cells, but not CD8 T cells or NK cells, from multiple sclerosis patients express NKG2C compared with controls. In addition, immunohistochemical analyses showed that multiple sclerosis brain tissues display HLA-E⁺ oligodendrocytes and NKG2C⁺ CD4 T cells. Our results implicate a novel mechanism through which infiltrating CD4 T cells contribute to tissue injury in multiple sclerosis. The Journal of Immunology, 2013, 190: 000–000.

Multiple sclerosis (MS) is an inflammatory disorder of the CNS characterized by the injury and destruction of oligodendrocytes and their myelin membranes. Both human studies and animal models of MS support the role of CD4 T cells in the pathogenesis of this disease (1). However, the mechanisms whereby CD4 T cells contribute to the distinct cell injury observed in MS have not been elucidated. Although oligodendrocytes in brain of MS patients express MHC class I molecules, they do not express MHC class II molecules, the TCR-CD4–specific ligand (2). Therefore, other target-recognition molecules are likely required for CD4 T cell–mediated injury of these myelin-producing cells. We (3) and other investigators (4) showed that a subset of human activated myelin-specific CD4 T cells acquires CD56, a marker associated with NK cells, and that such expression correlates with cytotoxic properties. These CD56⁺ CD4 T cells can kill human oligodendrocytes in a non-MHC–restricted manner, and such cytotoxicity is not inhibited by an anti-CD56 Ab, strongly supporting that other molecules underlie this effector function (3).

Human activated T cells can acquire NKRs, including members of the NKG2 family. NKG2A and NKG2C form heterodimers with CD94; both heterodimers (NKG2A/CD94 and NKG2C/CD94) recognize HLA-E, a nonclassical MHC class I molecule (5). HLA-E binding to the NKG2C/CD94 complex conveys an inhibitory signal. Expression of the NKG2C/CD94 complex was reported on human NK cells and T cells; either total CD3⁺ T cells or CD3⁺ CD8⁺ T cells were shown to bear this receptor (6–11). A subset of ex vivo human CD3⁺ T cells, obtained from the peripheral blood of healthy donors, express NKG2C/CD94⁺, mostly within the population that also CD56⁺; different groups reported that, on average, <5% of total CD3 T cells from healthy donors express NKG2C (6, 7). NKG2C is also observed on human T cells, mainly CD8 T cells, concurrently with other NKRs, such as NKG2D (7). The proportion of CD3⁺ T cells expressing the NKG2C/CD94 complex is elevated under pathologic conditions, such as human CMV infection (8, 9), active celiac disease (10), paroxysmal nocturnal hemoglobinuria (7), Stevens-Johnson syndrome, and toxic epidermal necrolysis (11). Moreover, NKG2C can mediate the expansion of infiltrating cytotoxic CD8 T cells in active celiac patients; celiac enterocytes in these patients express elevated levels of HLA-E, the cognate ligand (10). Similarly, NKG2C is detected on an elevated proportion of NK cells and CD8 T cells in blister fluids of patients with delayed cutaneous hypersensitivity reactions. These NK cells or CD8 T cells degranulate in response to keratinocytes expressing increased levels of HLA-E (11). Whether the interaction between NKG2C and its ligand HLA-E...
plays a role in the pathogenesis of MS has not been evaluated. Moreover, whether CD4 T cells can acquire this receptor in pathological conditions has not been shown.

In the current study, we provide evidence that NKG2C is expressed by human myelin-specific and short-term PHA-activated CD4 T cells. NKG2C is concomitantly expressed with CD56 and other molecules involved in cytotoxicity. We found that, in response to inflammatory cytokines, primary cultures of human oligodendrocytes upregulate HLA-E, the cognate ligand. We demonstrate that the cytotoxicity of PHA-activated or myelin-specific CD4 T cells toward human oligodendrocytes is, at least in part, mediated via NKG2C. We further show that a specific CD4 T cell population, which expresses NKG2C and other activating NKR's and has an enhanced cytotoxic capacity, is enriched in the peripheral blood of patients with MS compared with healthy controls. Finally, we establish that NKG2C+ CD4 T cells and HLA-E-expressing oligodendrocytes are present in the CNS of MS patients.

Materials and Methods

Donors
Seventeen patients with clinically definite MS according to the criteria of McDonald et al. (12) and characterized by a relapse–remitting (n = 8), secondary progressive (n = 5), or primary progressive (n = 4) disease course and 12 healthy volunteers were included in the study. None of the patients had received immunosuppressive, immunomodulatory, or steroid therapy for ≥6 mo prior to blood collection. Informed consent was obtained from all donors according to the local ethics committees (Centre Hospitalier de l’Université de Montréal or McGill University ethical boards).

Myelin-specific CD4 T cell lines

CD4 T cell lines specific for myelin basic protein (MBP) were expanded as previously described using whole human MBP (40 µg/ml) as Ag (13). Briefly, CD4 T cell lines were stimulated with autologous Ag-loaded B cells every 10–14 d, and recombinant human IL-2 (25 U/ml; Roche, Nutley, NJ) was added every 3 d between each round of stimulation. CD4 T cell lines were considered specific when the amount of IFN-γ secreted after 24–36 h (ELISA from BD Biosciences) in the presence of specific peptide–loaded autologous B cells was significantly greater (>1.5-fold) than the amount secreted in the presence of irrelevant peptide–loaded B cells.

Isolation and activation of human CD4 T cells

PBMCs were isolated by density gradient using Ficoll-Paque PLUS (GE Healthcare). PBMCs were either stained for flow cytometry analysis or cultured for 5 d at 2 × 10^6 cells/ml in the presence of anti-CD3 (0.9 µg/ml; BD Biosciences) Abs, PHA (2 µg/ml; Sigma-Aldrich), IL-2 (1000 U/ml; Roche), IL-12 (10 ng/ml, Biosource), or IL-15 (10 ng/ml; R&D Systems) in RPMI 1640 supplemented with 10% FBS, glutamine, and antibiotics (complete RPMI). CD4 T cells were purified from PHA-activated PBMCs using CD4 MicroBeads (Miltenyi Biotec), according to the manufacturer’s instructions. Purity was typically >98%, as assessed by flow cytometry.

Isolation and activation of adult human oligodendrocytes

CNS tissue was obtained from surgical resections performed for the treatment of nontumor-related epilepsy, in accordance with the guidelines set by the Biomedical Ethics Unit of McGill University. Oligodendrocytes were isolated from adult human brain as previously described (14) and maintained under basal culture conditions for 1 wk. The purity of these mature adult oligodendrocytes was characterized previously (15, 16); these cells express all mature myelin markers and do not proliferate. Oligodendrocytes were either left untreated or treated for 24 h with IFN-γ (1000 U/ml; Pierce Endogen), IL-1B (10 ng/ml; Invitrogen), and/or TNF (200 U/ml; Invitrogen).

Cytotoxicity assays

Oligodendrocytes were treated with IFN-γ (1000 U/ml) and IL-1B (10 ng/ml) for 24 h and then washed prior to the addition of CD4 T cells (2 × 10^5/2 ml at a 1:10 E:T ratio). Anti-CD94 (eBiosciences) or anti-NKG2D (M585 clone; kindly provided by Amgen) blocking Abs (25 µg/ml) or mouse IgG1 were added to CD4 T cells 1 h prior to their addition to target cells in the presence of 1 µM monomycin (Sigma-Aldrich) and anti-CD107a Ab (BD Biosciences) (17). After 8 h, effector cells were harvested and stained for NK2G2C and CD4. Oligodendrocytes were fixed with 2% (w/v) parafomaldehyde, blocked as described previously (18), and incubated with the cell-specific rabbit anti–Nogo-A Ab (10 µg/ml) for 1 h; finally, Alexa Fluor 488–conjugated goat anti-rabbit Ab (Invitrogen), which were added simultaneously with an anti-HLA-E Ab conjugated to PE (eBioscience). Mouse mAbs directed to human protein and conjugated to Alexa Fluor 488, Alexa Fluor 700, PE, PE-Cy7, Pacific Blue, or allophycocyanin were used to characterize immune cells. Surface stainings targeted CD3, CD4, CD8, CD56, NK2D, NK2A, NKp46 (all from BD Biosciences), NK2C, NKGL1, BioLex, from R&D Systems, and Fasl, Lrx, and Lymest from BD, intracellular staining targeted perforin (Abcam) and granzyme B (Invitrogen). Isotypes matched for control of primary antibodies were used for all stainings. All results were acquired on a LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar). Δ Median fluorescence intensity (MFI) was calculated by subtracting the fluorescence of the isotype from that of the stain.

Immunostaining of human brain samples

Immunostaining for NKG2C and CD4 was performed on snap-frozen sections, whereas HLA-E and O4 detection was performed on paraffin-embedded sections. Postmortem frozen brain sections from four patients diagnosed clinically and confirmed by neuropathological examination as having MS and one control were obtained from the NeuroResource tissue bank (University College London Institute of Neurology). Tissues were obtained from the tissue bank with informed consent following ethical review by the London Research Ethics Committee. Snap-frozen slices (10 µm thick) were air-dried, fixed in 4% parafomaldehyde for 10 min, and blocked for nonspecific binding for 1 h and then a mouse anti-human NK2G2C Ab (20 µg/ml; R&D Systems) was incubated 1 h at room temperature and then overnight at 4°C. Sections were then incubated for 1 h with a biotinylated goat anti-mouse Ab (Dako), followed by Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and immunoreactive rat anti-human CD3 (20 µg/ml; Lifespan Biosciences), followed by Alexa Fluor 488–conjugated donkey anti-rat Ab. Paraffin-embedded blocks from two patients diagnosed clinically and confirmed by neuropathological examination as having MS were analyzed. One patient was a 48-y-old female with chronic and chronic active plaques and diffuse disease affecting the white matter, the cortex, the brain stem, and the spinal cord. Patient 2 was a 51-y-old female with extensive toxic inactive central and white matter. Sections of 7 µm were stained with H&E and Luxol Fast Blue for histopathological examination and lesion localization. For immunostaining, sections were deparaffinized in toluene twice for 5 min each. Ag retrieval was done in sodium citrate (100°C, 30 min). Sections were blocked for 1 h and then incubated overnight with a mouse IgG1 anti–HLA-E Ab (20 µg/ml; R&D Systems) and a mouse IgM anti-O4 Ab (10 µg/ml; Chemicon), followed by a 1-h incubation with a mouse IgG1 anti–HLA-E Ab conjugated to Alexa Fluor 488 and Cy3-conjugated goat anti-mouse IgG1 (Jackson ImmunoResearch). Finally, all sections (paraffin and snap-frozen) were incubated with a nuclear stain TO-PRO-3 iodide (Invitrogen), treated with Sudan Black, and mounted as previously described (18). Controls were concomitantly performed on adjacent sections using appropriate primary isotype controls at the same concentrations. Slides were observed using a SPS Leica confocal microscope. Confocal images were acquired simultaneously in different channels throughout 4–8-µm z-stack every 0.2–0.5 µm using LAS AF software. We validated staining specificity by lack of signal only when the corresponding laser was turned off but not when others were still on. Moreover, we confirmed the absence of bleed-through by re-examining selected sections using sequential scanning. Images were subsequently merged using Adobe Photoshop software.

Statistical analyses

Statistical analyses were performed using Prism GraphPad software and included the Student t test or ANOVA followed by Bonferroni as post hoc test. Only p values < 0.05 were considered significant.
**Results**

Activated CD4 T cells expressing NKG2C display a cytotoxic profile

We (3) and other investigators (4) showed that human myelin-specific CD4 T cells expressing CD56 exhibit elevated cytotoxicity toward human oligodendrocytes compared with their CD56-negative counterparts. However, an anti-CD56-blocking Ab does not inhibit the CD56+ CD4 T cell–mediated cytotoxicity, which indicates that other molecules must be functionally required (3). Therefore, we assessed whether myelin-specific CD4 T cells could gain receptors associated with their cytotoxicity toward oligodendrocytes. Because NKG2C has been detected concurrently with CD56 on human T cells (7), we investigated whether this receptor plays a role in oligodendrocyte injury. We analyzed nine MBP-specific CD4 T cell lines expanded in vitro from the peripheral blood of two donors, according to our published protocol (13). We established that within each MBP-specific CD4 T cell line an average of 13% of the cells expressed NKG2C, and this receptor was concomitantly expressed with CD56 (Fig. 1A, 1B, top panels). When we analyzed CD56+ CD4 T cells specifically, we observed that the vast majority (>85%) displayed NKG2C but did not express NKG2A (Fig. 1A, 1B, bottom panels).

We determined whether other activation conditions could induce NKG2C on CD4 T cells. We assessed NKG2A and NKG2C expression on CD4 T cells under resting conditions and following a 5-d activation with cytokines (IL-15, IL-12, IL-2), TCR stimulation (anti-CD3 + anti-CD28 Abs), or PHA. We observed a minimal expression of NKG2C and NKG2A on ex vivo and untreated CD4 T cells obtained from healthy controls; these levels were not altered by cytokines or TCR triggering (Fig. 1C). However, PHA activation induced a significantly greater proportion of CD4 T cells expressing NKG2C (average 33%) for all tested donors (Fig. 1C). We analyzed both MBP-specific CD4 T cell lines (n = 4 from two donors) and short-term PHA-activated CD4 T cells (n = 3) for additional NK-associated markers (Fig. 1D). We compared NKG2C+ (top panels) and NKG2C− (bottom panels) cells and observed that most NKG2C+ cells did not have NKG2A on their surface. In contrast, elevated proportions of NKG2C+ cells expressed Nkp46, NKG2D, CD56, FasL, granzyme B, and perforin compared with their NKG2C− counterparts (Fig. 1D). These observations were confirmed on cells obtained from multiple donors (Fig. 1E). Overall, our results suggest that the acquisition of NKG2C by activated human CD4 T cells correlates with the expression of numerous molecules involved in cytotoxicity.

Human oligodendrocytes express HLA-E and are targeted by NKG2C+ CD4 T cells

Levels of HLA-E, the cognate ligand of NKG2C and NKG2A, are upregulated in various human cell types targeted during inflammatory disorders (10). Therefore, we investigated whether human oligodendrocytes express HLA-E, the ligand of NKG2C and NKG2A, under basal conditions or conditions mimicking the inflamed environment observed in the CNS of MS patients. Primary cultures of human oligodendrocytes under basal conditions

**FIGURE 1.** Activated NKG2C+ CD4 T cells display a cytotoxic profile. (A and B) The expression of CD56, NKG2A, and NKG2C by human MBP-specific CD4 T cell lines was assessed. (A) Representative FACS dot plots gated on living cells for the isotype controls or CD4+CD3+ T cells. (B) The percentage of CD4+CD3+ T cells (top panel) or CD56+CD4+CD3+ T cells (bottom panel) within each individual MBP-specific T cell line expressing NKG2C or NKG2A is illustrated for nine T cell lines obtained from two donors. A greater proportion of MBP-specific CD4 T cells express NKG2C compared with NKG2A. Moreover, most CD56+ MBP-specific CD4 T cells express NKG2C. (C) Representative FACS dot plots of PBMCs gated on CD3+ CD4+ T cells either ex vivo or following a short in vitro activation. Cells were not treated (Nil) or were treated with different stimuli: anti-CD3 + anti-CD28, PHA, IL-2, IL-12, or IL-15. Only PHA activation induced NKG2C expression on CD4 T cells. (D and E) The expression of molecules involved in cytotoxicity was assessed in four distinct MBP-specific CD4 T cell lines and PHA-activated CD4 T cells from three donors. (D) Representative FACS dot plots gated on CD4+CD3+ T cells that were NKG2C+ (top panels) or NKG2C− (bottom panels). (E) Percentage of NKG2C+ and NKG2C− PHA-activated CD4 T cells expressing NKG2D, CD56, Nkp46, FasL, and lytic enzymes. Data are mean ± SEM, *p < 0.05, **p < 0.01, NKG2C+ versus NKG2C−, Student t test (n = 3).
(nil) expressed detectable, but low, levels of HLA-E (Fig. 2A), as measured by flow cytometry. IFN-γ or IL-1β increased those levels; however, combined cytokines were more potent at inducing a significant upregulation of HLA-E ($p < 0.05$ versus nil) (Fig. 2A). We found that TNF, either alone or added to IL-1β or IFN-γ, had no impact on HLA-E expression (data not shown). Our results demonstrate that human oligodendrocytes enhance their low basal levels of HLA-E in response to pro-inflammatory cytokines, suggesting that immune mediators present in MS lesions (20, 21) could enhance such expression.

We then determined whether activated NKG2C+ CD4 T cells could target human oligodendrocytes expressing HLA-E. We used the CD107a mobilization flow cytometry–based assay, because we and other investigators showed previously that this assay correlates with the $[\text{51Cr}]$–release assay (22); CD107a is transiently detected on the cell surface of degranulating cytolytic cells. Using this assay, we simultaneously analyzed NKG2C+ and NKG2C- cells. To inhibit the interaction between NKG2C+ CD4 T cells and HLA-E on oligodendrocytes, we applied a commercially available blocking Ab targeting CD94, the coreceptor of NKG2A and NKG2C, as used by other investigators (23). CD4 T cells purified from PHA-activated PBMCs were preincubated with either an isotype control (Fig. 2B, left panels), an anti-CD94 (Fig. 2B, middle panels), or an anti-NKG2D (Fig. 2B, right panels) blocking Ab and then added to IFN-γ + IL-1β–treated oligodendrocytes in the presence of an anti-CD107a Ab or an isotype. After 8 h, CD4 T cells were harvested and analyzed by flow cytometry for the expression of NKG2C, CD4, and CD107a. We gated either on NKG2C+ (top panels) or NKG2C- (bottom panels) CD4 T cells and assessed the expression of CD107a (Fig. 2B). CD4 T cells in the absence of target cells did not degranulate significantly, with only 1% expressing CD107a. In contrast, we detected a significant

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**FIGURE 2.** NKG2C+ CD4 T cells target HLA-E–expressing human oligodendrocytes. (A) The expression of HLA-E was determined by flow cytometry on human oligodendrocytes that were not treated or were cultured in the presence of IFN-γ, IL-1β, or IFN-γ + IL-1β. Representative graphs of HLA-E expression on Nogo-A–gated oligodendrocytes (left panel). Fold increase in the HLA-E ΔMFI of oligodendrocytes from three donors (right panel; mean ± SEM). Treatment with either IFN-γ ($\xi p = 0.0623$) or IFN-γ + IL-1β ($\xi p < 0.05$) consistently elevated HLA-E expression by oligodendrocytes. (B and C) Cytotoxic potential of PHA-activated CD4 T cells and MBP–specific CD4 T cells toward IFN-γ + IL-1β–treated oligodendrocytes was evaluated using the CD107a assay after a 8-h coculture. (B) Representative dot plots of CD107a expression on PHA-activated CD4 T cells preincubated with an isotype control, an anti-CD94 blocking Ab, or an anti-NKG2D blocking Ab prior to their addition to oligodendrocytes (left panel). Flow cytometry events were gated on either CD3+CD4+ NKG2C+ or CD3+CD4+ NKG2C- cells. Data are from PHA-activated CD4 T cells obtained from three to six donors (mean ± SEM). The percentage of CD107a–expressing cells for effector cells alone or cells preincubated with an isotype control, an anti-CD94 blocking Ab, or both anti-CD94 and anti-NKG2D blocking Abs (right panel). (C) Oligodendrocytes were incubated in the absence (no effector) or in the presence of PHA-activated CD4 T cells preincubated with either an isotype control (Isotype) or anti-CD94 + anti-NKG2D blocking Abs, fixed, and stained for Nogo-A. Photomicrographs are representative of five fields from killing assays performed with two distinct CD4 T cell donors. Scale bar, 10 μm. (D) Representative dot plots of CD107a expression on MBP–specific CD4 T cells, preincubated with either an isotype control, an anti-CD94 or alone prior to their addition to oligodendrocytes. Flow cytometry events were gated on CD3+CD4+ cells. Data obtained from six MBP–specific CD4 T cell lines generated from two donors are shown (mean ± SEM). *$p < 0.05$, **$p < 0.01$, isotype versus anti-CD94, anti-NKG2D, or anti-CD94 + anti-NKG2D.
level of CD107a expression (19%) on activated NKG2C+, but not NKG2C−, CD4 T cells added to human oligodendrocytes in the presence of an isotype control. We further observed that blocking NKG2C via an anti-CD94 Ab led to a significant decrease in CD107a expression by NKG2C+ cells (9%). Degranulation by MBP-specific CD4 T cells preincubated with an anti-CD94 blocking Ab (Fig. 2D) before being added to inflamed oligodendrocytes was also significantly lower (22%) than what we observed on their counterparts preincubated with an isotype control (33%). CD4 T cells in the absence of target cells did not display significant CD107a (Fig. 2D, left panel). The significant impact of blocking the NKG2C/CD94 complex on MBP-specific CD4 T cells was confirmed using six distinct lines expanded from two donors (Fig. 2D). Overall, we established that blocking the NKG2C interaction with its ligand inhibited CD4 T cell–mediated killing of human oligodendrocytes (Fig. 2B–D). Note that because CD4 T cells in PHA-activated cells or MBP-specific CD4 T cells was confirmed using six distinct lines expanded from two donors (Fig. 2D). Overall, we established that blocking the NKG2C interaction with its ligand inhibited CD4 T cell–mediated killing of human oligodendrocytes (Fig. 2B–D). Note that because CD4 T cells in PHA-activated cells or MBP-specific lines showed undetectable/low levels of NKG2A, the anti-CD94 blocking Ab is presumed to interact with the NKG2C/CD94 heterodimers on these effector CD4 T cells.

Because we previously showed that oligodendrocytes are susceptible to NKG2D-mediated cytotoxicity because they express the cognate ligands (19), we also evaluated the contribution of this receptor. When the activated CD4 T cells were incubated with an anti-NKG2D blocking Ab (Fig. 2B) prior to their addition to oligodendrocytes, CD107a expression was reduced (11%) in the NKG2C+ population compared with the isotype control. Moreover, when PHA-activated CD4 T cells were preincubated with both anti-CD94 and anti-NKG2D blocking Ab, their degranulation levels were similar to those observed in the absence of target cells, demonstrating that the combined effect of NKG2C and NKG2D is responsible for most of their cytotoxic activity (Fig. 2B, right panel). Finally, we assessed the proportion of oligodendrocytes that survived the cocultures by immunocytochemistry; we observed that when effector cells were preincubated with an isotype control most oligodendrocytes died, whereas when the same effectors were preincubated with both anti-CD94 and anti-NKG2D blocking Abs, the proportion of oligodendrocytes was similar to the cells cultured in the absence of effector cells (Fig. 2C). These observations confirm that NKG2C and NKG2D contribute to most degranulation of effector cells and killing of oligodendrocytes.

NKG2C+ CD4 T cells are more abundant in MS patients and exhibit a cytotoxic profile

To evaluate the physiological relevance of NKG2C, we investigated NKG2A and NKG2C expression on ex vivo PBMCs from untreated MS patients and healthy controls by flow cytometry, using specific cell markers for CD4 (CD3+) and CD8 (CD3+) T cells and NK cells (CD3−CD56+). Representative dot plots from one MS patient and one healthy control for NKG2C expression on gated CD4 T cells, CD8 T cells, or NK cells demonstrate that NKG2C was detected only on small subsets in healthy controls (Fig. 3A). In contrast, a greater proportion of CD4 T cells expressed NKG2C in MS patients compared with healthy controls (9.6 versus 3.0%); results from 17 untreated MS patients and 12 normal controls show a significantly higher percentage of NKG2C+ CD4 T cells in MS patients (Fig. 3B). In contrast, the proportion of CD8 T cells and NK cells positive for NKG2C was similarly low in patients and normal controls (Fig. 3A, 3B). These increased proportions of NKG2C+ CD4 T cells in MS patients were observed regardless of their clinical status (relapsing–remitting versus secondary progressive MS). We assessed samples from eight relapsing–remitting MS, four primary progressive MS, and five secondary progressive MS patients by flow cytometry. PBMCs obtained from untreated MS patients (MS) or normal controls (NC) were analyzed ex vivo for immune molecules. (A) Representative dot plots of events gated on living cells for isotype control or stained samples: CD3+CD4+CD8− (CD4 T cells), CD3+CD4−CD8+ (CD8 T cells), or CD3−CD56− (NK cells). One typical example of a control and one MS patient are shown. (B) The percentage of NKG2C expression on CD4 T cells, CD8 T cells, and NK cells observed in blood samples obtained from 17 MS patients and 12 normal controls. (C) Representative dot plots of CD3+CD4+CD8− T cells from untreated MS patients gated on NKG2C+ (top panels) or NKG2C− (bottom panels) cells for the expression of NKG2A, NKG2D, CD56, Nkp46, FasL, granzyme B, and perforin. (D) Pool data obtained from four untreated MS patients; results are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, NKG2C+ versus NKG2C−, Student t test (n = 4).

FIGURE 3. NKG2C+ CD4 T cells are more abundant in MS patients and exhibit a cytotoxic profile. PBMCs obtained from untreated MS patients (MS) or normal controls (NC) were analyzed ex vivo for immune molecules. (A) Representative dot plots of events gated on living cells for isotype control or stained samples: CD3+CD4+CD8− (CD4 T cells), CD3+CD4−CD8+ (CD8 T cells), or CD3−CD56− (NK cells). One typical example of a control and one MS patient are shown. (B) The percentage of NKG2C expression on CD4 T cells, CD8 T cells, and NK cells observed in blood samples obtained from 17 MS patients and 12 normal controls. (C) Representative dot plots of CD3+CD4+CD8− T cells from untreated MS patients gated on NKG2C+ (top panels) or NKG2C− (bottom panels) cells for the expression of NKG2A, NKG2D, CD56, Nkp46, FasL, granzyme B, and perforin. (D) Pool data obtained from four untreated MS patients; results are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, NKG2C+ versus NKG2C−, Student t test (n = 4).
progressive MS; the average percentage of NKG2C+ CD4 T cells in each group was 8.3, 9.3, and 8.5%, respectively. NKG2A was not detected on ex vivo T cells but only on a subset of NK cells, and no difference was observed between MS patients and healthy controls (data not shown).

We compared NKG2C+ and NKG2C− CD4 T cells obtained from MS patients for the expression of multiple immune effector molecules. Representative dot plots are shown and demonstrate the differences between these subsets (Fig. 3C). A minor or undetectable population expressed NKG2A in both subpopulations. However, a greater proportion of NKG2C+ CD4 T cells expressed Kp46, NKG2D, CD56, FasL, granzyme B, and perforin compared with the NKG2C− cells. We confirmed the cytotoxic profile of NKG2C+ CD4 T cells compared with the NKG2C− counterparts on ex vivo blood samples from three MS patients (Fig. 3D). We observed that the ΔMFI (∼1300) for NKG2C on ex vivo CD4 T cells from MS patients was similar to that on short-term PHA-activated CD4 T cells from healthy donors. Overall, these results show that a subpopulation of CD4 T cells expressing NKG2C and cytotoxicity-associated immune effector molecules is more abundant in the peripheral blood of MS patients compared with healthy controls.

**HLA-E+ oligodendrocytes and NKG2C+ CD4 T cells are present in MS brain tissue**

We next sought to determine whether the HLA-E+ NKG2C interaction takes place in the CNS of MS patients using immunohistochemistry on postmortem CNS tissues. We observed in paraffin-embedded sections obtained from three MS patients that cells of oligodendrocyte lineage (O4+ cells) can express HLA-E in areas from lesions involving both white matter and cortex (Fig. 4A–C). Hence, we can conclude that human oligodendrocytes both in vitro (Fig. 2) and in situ (Fig. 4) express HLA-E, especially in inflammatory conditions. We also detected cells expressing HLA-E that were negative for O4, suggesting that cells other than oligodendrocytes could express this ligand (Fig. 4C, white arrows). However, similarly to a recent report (24) we could not colocalize GFAP and HLA-E (Fig. 4D), suggesting that there are HLA-E-expressing cells in MS brain tissues that are neither oligodendrocytes nor astrocytes. We observed HLA-E expression on blood vessels (data not shown) as demonstrated previously (24). Conversely, within frozen tissues obtained from four distinct brain samples from three MS patients, we found a subset of CD4 T cells (12%) both in perivascular and parenchymal areas that expressed NKG2C (Fig. 4E, orange arrowheads). The proportion of NKG2C+ CD4 T cells was similar to the percentage of CD4 T cells expressing NKG2C that we observed in the peripheral blood of untreated MS patients (9.6 ± 1.3%). We also detected NKG2C+ cells that did not carry CD4 but displayed a morphology of infiltrating leukocytes, suggesting that other infiltrating immune cells express NKG2C (Fig. 4E, white arrows). We could not detect NKG2C+ CD4 T cells in our CNS sections from controls (data not shown). We were unable to successfully detect NKG2C on paraffin-embedded material and, conversely, HLA-E detection was unsuccessful on frozen samples; therefore, it was not possible to colocalize NKG2C and HLA-E on the same tissues. Despite this limitation, our results indicate that NKG2C+ CD4 T cells are more abundant in the peripheral blood of MS patients and infiltrate the inflamed CNS of these patients.

**Discussion**

In the current study, we identify a novel mechanism by which a specific subset of CD4 T cells in MS patients can directly interact and kill human oligodendrocytes. MS patients carry elevated proportions of NKG2C+ CD4 T cells (9.6 ± 1.3%) in their peripheral blood compared with healthy controls (3.0 ± 0.6%). These NKG2C+ CD4 T cells (Fig. 3) display enhanced effector functions by producing higher levels of lytic enzymes and increased expression of activating receptors, such as NKG2D, Nkp46, and FasL, compared with their NKG2C− counterparts. Similarly, NKG2C+ CD4 T cells observed in PHA-activated cells or in MBP-specific CD4 T cell lines exhibit elevated expression of these immune mediators (NKG2D, FasL, granzyme B, perforin), as well as an enhanced cytotoxic capacity toward human oligodendrocytes, compared with their NKG2C-negative counterparts (Figs. 1, 2). Blocking NKG2C or NKG2D on these activated CD4 T cells significantly decreased the targeting of human oligodendrocytes (Fig. 2). Moreover, our results provide the mechanism behind the previously observed cytotoxicity of CD56+ MBP-specific CD4 T cells toward oligodendrocytes (3); such cytotoxicity is, in part, through NKG2C, expressed concomitantly with CD56 (Fig. 2). Finally, we detected NKG2C+ CD4 T cells in MS lesions, supporting the notion that these cytotoxic cells are present in the inflamed CNS of these patients and can interact with HLA-E–expressing oligodendrocytes detectable in these tissues (Fig. 4).

Numerous observations demonstrated that CD4 T cells can be involved in the initiation and progression of MS in humans and experimental autoimmune encephalomyelitis models (1, 25). CD4 T cell infiltration is observed in MS lesions (26), and activated CD4 T cells can kill human oligodendrocytes in vitro (3). However, oligodendrocytes do not express detectable MHC class II in primary cultures (27) or in human brain sections from MS patients or control donors, although such expression is observed on microglia and macrophages (2, 28). Therefore, the mechanisms by which human CD4 T cells can exert cytotoxic effects on oligodendrocytes is most likely MHC class II independent. Indeed, we showed previously that PHA-activated and/or myelin-specific CD4 T cells mediate injury to human oligodendrocytes via MHC-unrestricted, CD56-independent, and TNF-independent mechanisms (29).

The concurrent expression of NKG2C and CD56 by a small subset of activated T cells was observed in healthy donors by other investigators (average reported: 0–5% of T cells) (6, 7, 30, 31), similar to what we observed (Fig. 3, 3.0 and 3.4% for CD4 and CD8 T cells, respectively); increased proportions of such cells were reported previously under pathologic conditions such as infection and inflammatory disorders (7–11). However, such increased percentage of lymphocytes expressing NKG2C is observed predominantly in CD8 T cells or NK cells compared with healthy controls (7, 11). The proportion of CD8 T cells expressing NKG2C was reported to be slightly increased in untreated MS patients (3.0%) compared with controls (2.0%) (32). However, we observed a comparable low level of CD8 T cells expressing NKG2C in both groups (3.6% in MS patients compared with 3.4% in controls). In contrast to these previous reports, we find a selective increase in NKG2C-expressing cells only among CD4 T cells in MS patients (Fig. 3).

CD8 T cell clones expanded in vitro and carrying regulatory functions against myelin-specific CD4 T cell clones were reported to express elevated levels of NKG2A when expanded in vitro from blood samples obtained from MS patients compared with those from healthy controls (33). NKG2A+ CD8 T cells carrying regulatory functions were also shown in the MS animal model, experimental autoimmune encephalomyelitis (34). In contrast, Martinez-Rodriguez et al. (32) reported a similar proportion of CD8 T cells carrying NKG2A for controls and untreated MS patients and of NK cells expressing NKG2A and/or NKG2C. Similarly, we rarely found NKG2A+ T cells when we performed ex vivo analysis of blood cells and could not detect any differences between MS patients and controls (Fig. 3). We can speculate
that the discrepancy between the observations made on in vitro-expanded cells (33) and the ex vivo detection of NKG2A by our group (Fig. 3) and other investigators (32) on CD8 T cells might be due to in vitro–induced effects.

NKG2C+ CD4 T cells, either ex vivo from MS patients or within MBP-specific or PHA-activated CD4 T cells, express molecules linked to cytotoxicity. A greater proportion of these cells display FasL. Human oligodendrocytes express elevated levels of Fas in MS lesions (14); therefore, infiltration of these NKG2C+ CD4 T cells into the CNS could potentially exacerbate oligodendrocyte damage. We previously observed a partial inhibition of CD56+ CD4 T cell–mediated killing of human oligodendrocytes upon the addition of an anti-Fas Ab (3). Moreover, other investigators showed that MBP-specific CD4 T cells can mediate their destruction of oligodendrocytes in vitro (41).

**FIGURE 4.** HLA-E and NKG2C are expressed in MS brain tissues. (A) Representative staining of paraffin-embedded sections with Luxol Fast Blue for myelin. Cortex (C) and white matter (WM) are identified; within the white matter, well-demarcated MS lesions (L) are seen, as well as diffuse areas of demyelination and tissue damage, known as dirty appearing white matter (DAWM). Some areas of cortex contain cortical demyelinated lesions (CL). (B) H&E-stained section of the area of DAWM depicted in (A); tissue is rarefied due to loss of myelin, reactive astrocytes (black arrows) are seen throughout the area, and a mononuclear infiltrate (blue arrowheads) is observed in the perivascular space around a blood vessel. Original magnification ×200. (C) The MS brain tissue characterized in (A) and (B) was also immunostained for HLA-E (green), O4 (red), and nuclear stain (blue). Fluorescent microscopy shows the presence of oligodendrocytes (O4+) expressing HLA-E. Orange arrowheads indicate examples of oligodendrocytes expressing HLA-E; white arrows indicate an HLA-E–expressing cell that is not of oligodendrocyte lineage. Areas in white boxes are shown enlarged in the far right column, together with the corresponding isotype controls (original magnification ×3500). Data shown are representative of 15 fields from three distinct MS tissue blocks. Scale bars, 25 μm. (D) An acute MS lesion was also immunostained for HLA-E (red), GFAP (green), and nuclear stain (blue). HLA-E+ cells could be detected, but these cells are not astrocytes (GFAP+). White arrows indicate examples of HLA-E–expressing cells that are not of astrocyte lineage. Area in white box is shown enlarged in the far right column, together with the corresponding isotype control. Original magnification ×400. (E) Frozen brain tissues from patients with MS were immunostained for CD4 (green), NKG2C (red), and nuclear stain (blue). Fluorescent microscopy reveals the presence of CD4 T cells and NKG2C+ cells. Orange arrowheads indicate examples of NKG2C+CD4 T cells, white arrows indicate an example of an NKG2C+ cell that is not a CD4 T cell, and pink arrows indicate examples of CD4 T cells that do not express NKG2C. Isotype controls and enlarged areas in white boxes are shown in the last column (original magnification ×2500–×3500). Three distinct fields obtained from two MS brain specimens, representative of four distinct MS brain samples, are shown. Scale bars, 25 μm.
cytotoxicity through Fas–FasL interactions and perforin release (35). The NKG2C+ CD4 T cells also express NKG2D, another receptor involved in cytotoxicity. NKG2D blockade partially abrogated their cytotoxic potential upon contact with oligodendrocytes (Fig. 2), similar to what we previously reported for activated human NK cells, γδ T cells, and CD8 T cells (19). Indeed, we showed that oligodendrocytes both in vitro and in post-mortem samples express ligands of NKG2D (19). Moreover, cytotoxic CD4 T cells expressing NKG2D were detected at the inflammation site of other pathologies, such as the inflamed intestine of patients with Crohn’s disease (36) and the synovial tissue of patients with rheumatoid arthritis (37). In each case, NKG2D+ CD4 T cells were associated with tissue destruction. Because concurrent blockade of NKG2C and NKG2D completely abrogated CD4 T cell degranulation and oligodendrocyte killing (Fig. 2), recognition of their cognate ligate by these receptors is most likely responsible for most of the cytotoxicity mediated by these cells. Our phenotyping results suggest that other activating receptors, such as NKp46, could also contribute to CD4 T cell-mediated cytotoxicity, although the cognate ligand remains unknown. Interestingly, NKp46 was detected on lymphoid cells in demyelinated areas, especially in active MS lesions, but colabeling has not been performed to confirm whether these cells are NK cells or T cells (38). Finally, analysis of a small subset of donors (data not shown) suggests that NKG2C+ CD4 T cells are mainly effector memory cells, being CCR7+ as reported by others (7).

Elevated levels of HLA-E (or its mouse equivalent Qa-1) can modulate the susceptibility of target cells to the injury mediated by effector cells bearing the cognate receptors NK2A or NKG2C. Murine Qa-1+ neurons are protected from the cytotoxicity mediated by NK2A+ CD8 T cells (39), whereas HLA-E+ trophoblast cells that surround a developing embryo are protected from NKG2A+ NK cells (40). We also observed that the HLA-E expression by human oligodendrocytes decreased NK cell–mediated cytotoxicity (data not shown). Conversely, in celiac disease, the inflamed bowel induces the expansion of NKG2C+ CD8 T cells that target intestinal HLA-E+ enterocytes (10). Moreover, upregulated expression of HLA-E was observed in epidermal keratinocytes from the affected skin of patients with drug-induced delayed hypersensitivity reactions, and these HLA-E–expressing keratinocytes are susceptible to NKG2C+ CD8 T cell–mediated killing (11). Because we observed the presence of both NKG2C+ CD4 T cells and HLA-E–expressing oligodendrocytes in the CNS of MS patients (Fig. 4), the interaction between NKG2C+ CD4 T cell effectors and HLA-E–target cells likely occurs in vivo in the target tissue of this disease. Lastly, our data suggest a second population of NKG2C+ cells (separate from CD4 T cells) that could also participate in oligodendrocyte vulnerability (Fig. 4); additional studies will be needed to characterize this population.

In summary, we demonstrate a novel contributing mechanism by which pathogenic CD4 T cells expressing NKG2C can directly target HLA-E–expressing human oligodendrocytes in the context of MS. Some approved MS therapies targeting immune molecules (e.g., α4-integrin) that are broadly expressed by human lymphocytes have led to serious infections in some patients (41). In contrast, because NKG2C is specifically expressed by a subset of cytotoxic CD4 T cells in MS patients, targeting this receptor would not affect large populations of immune effector cells. Therefore, the development of therapies directed at NKG2C warrant further investigations.

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Disclosures

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References

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