



KIR2DL4-HLAG interaction at human NK cell-oligodendrocyte interfaces regulates IFN- γ -mediated effects

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ABSTRACT

Interactions between germline-encoded natural killer (NK) cell receptors and their respective ligands on tumorigenic or virus-infected cells determine NK cell cytotoxic activity and/or cytokine secretion. NK cell cytokine responses can be augmented in and can potentially contribute to multiple sclerosis (MS), an inflammatory disease of the central nervous system focused upon the oligodendrocytes (OLs). To investigate mechanisms by which NK cells may contribute to MS pathogenesis, we developed an *in vitro* human model of OL-NK cell interaction. We found that activated, but not resting human NK cells form conjugates with, and mediate cytotoxicity against, human oligodendrocytes. NK cells, when in conjugate with OLs, rapidly synthesize and polarize IFN- γ toward the OLs. IFN- γ is capable of reducing myelin oligodendrocyte and myelin associated glycoproteins (MOG and MAG) content. This activity is independent of MHC class-I mediated inhibition via KIR2DL1, but dependent upon the interaction between NK cell-expressed KIR2DL4 and its oligodendrocyte-expressed ligand, HLA-G. NK cells from patients with MS express higher levels of IFN- γ following conjugation to OLs, more actively promote *in vitro* reduction of MOG and MAG and have higher frequencies of the KIR2DL4 positive population. These data collectively suggest a mechanism by which NK cells can promote pathogenic effects upon OLs.

1. Introduction

Oligodendrocytes (OLs), glial cells of the central nervous system (CNS), create insulating myelin sheaths that surround and support neuronal axons. This insulation allows electrical conduction to move rapidly along the length of a neuron, passing from one uninsulated area (node of Ranvier) to another. Focal interruption of myelin sheaths and the resultant electrical impulse slowing are hallmarks of multiple sclerosis (MS). MS is an inflammatory and autoimmune disease of the CNS, in which adaptive immunity is believed to play a major role in disease progression (Sospedra and Martin, 2005). Existing studies also

uphold at least some regulatory and/or pathogenic role for components of the innate immune system, including NK cells, in MS pathogenesis (Kastrukoff et al., 1998; Takahashi et al., 2001; Kastrukoff et al., 2003; Takahashi et al., 2004; Trachtenberg, 2009; Gandhi et al., 2010; Gross et al., 2016a, b; Smith et al., 2017). However, the mechanism by which human NK cells contribute to the pathogenesis of MS is unclear.

NK cells function primarily to survey for and kill tumorigenic and virus infected target cells. In particular, they serve a pivotal role in the control of herpesvirus infection (Biron et al., 1999). Despite ongoing controversies (Simpson et al., 2014), there is also evidence for correlation between herpes viral infection and MS (Challoner et al., 1995;

Abbreviations: eNK cell, *ex vivo* natural killer cell; OL, oligodendrocyte; MAG, myelin-associated glycoprotein; MOG, Myelin oligodendrocyte glycoprotein; KIR2DL4, killer cell immunoglobulin like receptor, two Ig domains and long cytoplasmic tail 4; HLAG, human leukocyte antigen G; KIR2DL1, killer cell immunoglobulin like receptor, two Ig domains and long cytoplasmic tail 1; IFN- γ , γ interferon gamma; MS, multiple sclerosis; WT-KIR2DL4, WT KIR2DL4-GFP transduced NK92 cells; RG-KIR2DL4, RG mutant of WT KIR2DL4-GFP transduced NK92 cells

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Ablashi et al., 1998; Alvarez-Lafuente et al., 2002; Soldan et al., 1997; Pormohammad et al., 2018; Saberi et al., 2018). In light of these historical connections, a role for NK cells in MS pathogenesis that depends on the activation status of NK cells is possible. Additional support for this concept includes elevated levels of IL-2 in MS patient serum (Gallo et al., 1989) which would activate NK cells, as well as that *in vitro* activation of NK cells induces receptor-ligand dependent cytotoxicity against healthy autologous and heterologous OLS (Morse et al., 2001; Antel et al., 1998; Saikali et al., 2007).

Thus, we attempted to model the cellular interactions potentially occurring during the development of MS with a focus upon a role for NK cells (Rodriguez-Martin et al., 2015; Macchi and Mastino, 2016; Lagumersindez-Denis et al., 2017; Moreno-Torres et al., 2018) through an *ex vivo* human co-culture system using human NK cells and OLS. We also directly evaluated NK cells from MS patients to further consider how NK cells could potentially contribute to the pathogenesis of MS. We have identified some *in vitro* and direct HLA-independent activity by NK cells against OLS dependent upon specific receptor-ligand interactions. Furthermore the studies of patient NK cells directly suggest opportunities to strategize clinically for this challenging clinical condition.

2. Methods

2.1. Isolation of peripheral blood mononuclear cells (PBMCs) and primary natural killer (NK) cells from humans

Using Ficoll Hypaque (Amersham), PBMCs were isolated from healthy volunteers or patients with MS. *Ex vivo* NK (eNK) cells were isolated from PBMCs by negative selection using a human NK cell isolation kit (Miltenyi Biotec). NK cell preparations (CD3⁻ CD56⁺), as determined by flow cytometry (FACSCalibur, BD), were > 95% pure with < 1.0% CD3⁺ cells. Resting or IFN- α [1000 IU, 24 h, 37 °C, 5% CO₂] activated eNK cells were used as effector cells. For cytotoxicity assays and to induce KIR2DL4 expression, eNK cells were activated with 100 IU/ml of IL-2 (Orange et al., 2011) for 5 days.

2.2. Differentiation of human oligodendrocytes (OLs) from human oligodendrocyte precursor cells (HOPC)

Immature neuroepithelial (Nestin) and oligodendroglial (A2B5) marker positive human oligodendrocyte precursor cells (HOPC; ScienCell Research lab) (Raff et al., 1983) were maintained and differentiated into oligodendrocytes (OLs) as per the supplier's instructions. Antibodies against myelin-associated glycoprotein (MAG; Epitomics) and myelin oligodendrocyte glycoprotein (MOG; AnaSpec) were used to monitor OL differentiation at regular intervals. Differentiated OLs were used as targets. In some experiments, OLs were treated with 10 or 20 ng/ml of recombinant human IFN- γ (eBioscience) for 2.5 h at 37 °C.

2.3. Cell lines

The immortalized human NK cell line NK92 and an erythroleukemic target cell line K562 were grown and maintained in culture as described previously (Banerjee et al., 2007). GFP-expressing NK92 cells were generated by retroviral transduction (Banerjee et al., 2007). Briefly, 2 μ g of pBMN-internal ribosome entry site (IRES)-EGFP plasmid DNA were transfected into the Phoenix packaging line using Fugene (Roche) lipofection reagent. Supernatant was harvested on day 2 post-transfection. NK92 cells, Polybrene (Sigma), and supernatant were mixed and spun in a well of a 6-well plate at 1000 \times g for 90 min at 32 °C. Following overnight incubation at 32 °C, cells were spun down and resuspended in supplemented Myeolocult media and sorted for GFP expression. NK92 cells were also transduced to express FLAG-tagged WT or transmembrane arginine to glycine (RG)-mutant KIR2DL4

(Kikuchi-Maki et al., 2003; Miah et al., 2008) from the retroviral pBMN-IRES-EGFP vector. MO3.13 human oligodendroglial target cells (Buntinx et al., 2003) (CELLutions biosystem) were cultured in DMEM with 10% FCS (GIBCO); these were differentiated into mature OLS in serum-free media (Barbarese et al., 1988) after 15 days of culture. In some experiments MO3.13 cells were transfected with HLA-Cw3 and HLA-Cw4 cDNA (RIKEN BRC BioResource Center), which were commercially tagged to GFP (Epoch Life Sciences). For this, 1 \times 10⁶ of MO3.13 cells were transfected with 500 ng of DNA/ transfection, using 4D nucleofector kit SE, and DS126 program in a 4D nucleofector (Lonza). 24 h after transfection GFP-expressing cells were sorted and selected in G418 containing media. G418 concentration (2 mg/ml) was previously determined by using a death curve on untransfected cells.

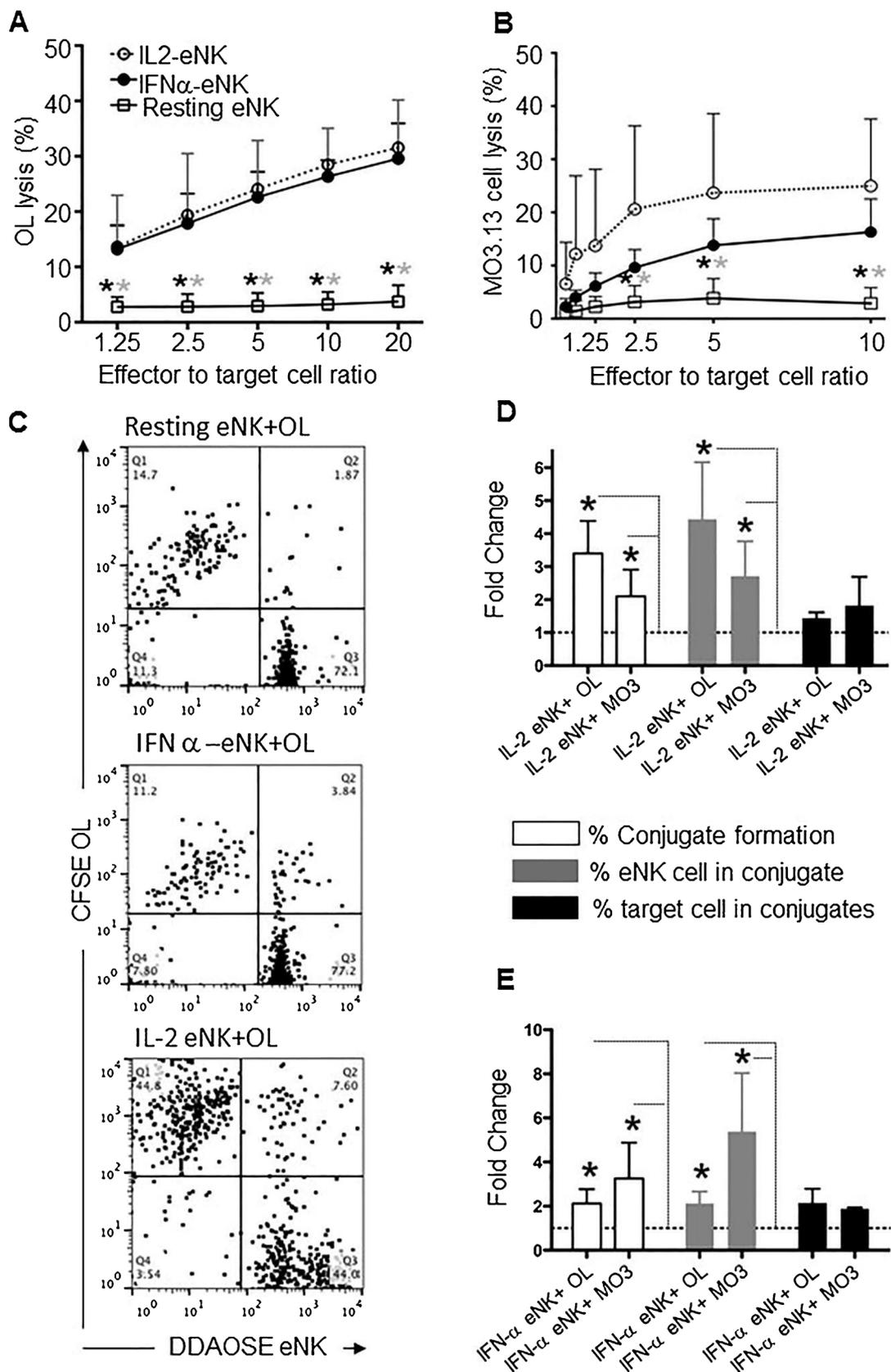
2.4. Flow cytometry-based assays

Resting or cytokine activated-eNK cells were labeled with DDAOSE (Invitrogen), while OLs, MO3.13 or K562 target cells were labeled with CFSE (Invitrogen) at 1.5 mM and 1.25 mM concentrations respectively per the manufacturer's instruction. Following labeling, effector and target cells were washed in PBS; labeling was quenched in RPMI with 10% FCS. Conjugates between DDAOSE -labeled eNK cells and CFSE-labeled target cells were formed at 2:1 effector to target cell ratio in 200 μ l of RPMI with 10% FCS for indicated time periods in a humidified CO₂ incubator at 37 °C. Cells were fixed in 2% paraformaldehyde and evaluated by flow cytometry (FACScalibur or Fortessa, BD Biosciences). FlowJo software (TreeStar) was used to identify single-color-positive unconjugated cells and dual-color-positive effector cell + target cell conjugates. To evaluate eNK cell activity in terms of reducing MAG and MOG content from OLs in MHC independent manner, dual-color-positive effector + target cell conjugates were evaluated. For this, HLA-Cw3 or -Cw4-positive MO3.13 cells were monitored through GFP fluorescence, and eNK cells were labeled with PKH26 (Sigma). In some experiments GFP positive NK92 cells or CFSE-labeled eNK cells were conjugated with DDAOSE-labeled MO3.13 cells. After 2.5 h of conjugate formation, cells were fixed and permeabilized in a perm-fix solution (BD Pharmingen), washed twice in perm-wash buffer (BD Pharmingen), blocked with 20 μ g/ml goat IgG (Santacruz) for 20 min at room temperature, washed again in a perm-wash buffer, and incubated with rabbit anti-human MOG (Anaspec) or rabbit-anti-MAG (Abcam) for 30 min at room temperature. F(ab')₂ specific human adsorbed fluorescent tagged anti-Rabbit IgG (Cederlane) was used as the secondary antibody to detect binding of primary rabbit antibodies in OLs and MO3.13 cells.

In some experiments, IFN- γ -expression in conjugated effector cells was measured after indicated time period of conjugate formation using PE conjugated anti-IFN- γ (BD Pharmingen). A mixture of anti-HLA-G monoclonal (Clone 87 G, Biolegend) and polyclonal (D-20, Santacruz) antibodies, each at 20 μ g/ml concentration, was added to block binding of HLAG to KIR2DL4. The polyclonal anti-HLAG antibody was used for flow cytometry-based staining of OLs and MO3.13 cells, and an anti-HLA-C antibody (clone DT9, EMD Millipore) was used to determine the expression of HLA-Cw3 and Cw4 on MO3 cells. Fluorescent-conjugated antibodies against CD antigens, NK cell surface receptors, and isotype-matched controls were purchased from BD Pharmingen. Allophycocyanin-tagged anti-human CD158d mAb (clone 33) was obtained from Biolegend. Singlet cells from lymphocyte aggregates were further gated on CD56⁺, CD3⁻ NK cell population using FlowJo (TreeStar) to determine percentages of NK cell and the intensity of, a given marker on them. Effects of anti-IFN- γ (eBioscience) and all other neutralizing antibodies were evaluated in respect to control antibodies at comparable concentrations.

2.5. Western blot analysis

Cells were lysed in ice-cold lysis buffer containing 25 mM Tris-Cl



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Fig. 1. Activated but not resting eNK cells mediate cytotoxicity against and form conjugates with oligodendrocytes and human oligodendrocytic MO3.13 cells. Cytotoxic activity of IL-2- (IL2-eNK, open circle), IFN- α – (IFN α -eNK, filled circle) treated, and control (resting, square) eNK cells against (A) oligodendrocytes (OLs) or (B) MO3.13 target cells was measured in 4 h ^{51}Cr -release assays. Each point represents the mean of 3 independent experiments; error bars indicate SD. Stars denote a statistically significant difference ($*P < 0.05$) between the cytotoxic activity of resting and IL-2 (gray stars) or IFN α -treated (black stars) eNK cells. (C) Representative examples of flow cytometry-based conjugation assays using CFSE-labeled OLs and DDAOSE-stained eNK cells, which were resting, IL-2- or IFN- α -treated prior to 30 min of conjugation at 37 °C. DDAOSE+ /CFSE+ conjugates appear in the upper right quadrant of each graph. (D, E) Quantitative analysis of conjugation between eNK cells and OLs or MO3.13 cells in 3 independent flow cytometry-based assays comparing the effect of IL-2- (D) or IFN- α - (E) activated with untreated (resting) eNK cells (dotted lines). Bars represent the mean fold increase of the total percentage of cells in conjugates (% Conjugate formation, open bars), percentages of NK cells in conjugates (% eNK cell in conjugate, gray bars), and percentages of target cells in conjugates (% target cell in conjugate, black bars), as noted. Error bars indicate SD; statistically significant fold increases over resting eNK cell + target cell conjugates (dotted line) are noted ($*P < 0.05$).

(pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 1 mM DTT and 5% glycerol. Protein estimation was performed using a bicinchoninic acid assay protein estimation kit (Pierce). 10 μg of cellular proteins (as indicated) was separated on 4–12% Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes (Invitrogen), blocked in 3% BSA and 140 mM NaCl-containing buffer, and then incubated with a primary antibody (Banerjee et al., 2007). Primary antibodies against MAG (Abcam), MOG (Anaspec) and β -actin (Sigma) were used. Bound antibody was detected with anti-rabbit, anti-mouse or anti-goat IgG coupled to fluorophores (AlexaFluor 680, Molecular Probes; IRDye 800, Li-Cor Biosciences), then imaged using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Densitometric analysis of protein bands was performed using ImageJ software.

2.6. Confocal microscopy and image analysis

Cells were prepared for evaluation of fixed conjugates by immunofluorescent confocal microscopy as previously described (Banerjee et al., 2007). Briefly, conjugates between effector and target cells were formed at 2:1 effector to target cell ratio in 200 μl of RPMI with 10% FCS for indicated time periods in a humidified CO₂ incubator. Conjugates or unconjugated target cells, as indicated, were adhered to poly-L-lysine-coated glass slides (Polyprep; Sigma-Aldrich) for 15 min, all at 37 °C. Slides were rinsed in PBS and cells were fixed and permeabilized with 4% formaldehyde, 0.1% saponin, and 0.1% Triton X-100 in PBS for 15 min, rinsed in PBS, and incubated at RT for 1 h with primary antibody, followed by secondary antibody as indicated.

In some experiments glass-adhered cells were incubated with anti-KIR2DL4 primary antibody, at 4 °C in PBS with 2% FCS and 0.05% sodium azide (Sigma), then washed and stained with secondary antibody in the same buffer and condition before the fixing and permeabilization process, which was required for IFN- γ staining. Anti-human primary antibodies against MOG (Anaspec), KIR2DL4 (Clone 181703, R & D Systems), HLA-G (D-20, Santacruz) and highly cross-adsorbed Alexafluor-fluorescent-tagged secondary antibodies (Molecular Probes) were used in the range of 1–20 $\mu\text{g}/\text{ml}$. Where specified, conjugates were also stained with FITC tagged anti-human perforin (BD) and Alexafluor 647-conjugated phalloidin or anti-IFN- γ antibody (BD). Stained cells were mounted in glycerol-based mounting media (Vectashield) and visualized using a spinning disc confocal microscope (IX-81, Olympus) under a 60x UPLFLN objective with a numerical aperture of 1.45 at 25 °C. Two-dimensional images were captured using an electron multiplying charged-coupled device camera (Hamamatsu, C9100). Image acquisition, 3-dimensional reconstitution and analysis of images were performed using Volocity software (PerkinElmer) (Banerjee and Orange). F-actin was quantified by multiplying the mean fluorescent intensity (MFI) of the phalloidin by its volume occupied within a single cell (Banerjee and Orange). OL volume was determined by selecting the region surrounding the cell as determined via fluorescent staining of OL cortical F-actin. The following formulas were used for image analysis: (a) MOG amount per OL = (volume occupied by MOG specific fluorophore / OL volume) \times (the MFI of MOG fluorophore); (b) Average MOG volume = (total volume of MOG from n number of OLs / n). Effector cell + target cell conjugates were also evaluated for overlapping areas of KIR2DL4 (NK92 cells) and HLA-G (MO3.13 cells). The

area of overlap was defined as the interface. The percentage of accumulation of a given protein at the interface was defined as (area \times MFI of a protein at the interface) / (area \times MFI of the same protein inside the cell) \times 100.

2.7. Cytokine array

Supernatants from various types of effector cell + target cell conjugates, and also from unconjugated cells were obtained after 16 h of incubation. Multiplex cytokine assays were performed at the Human Immune Monitoring Center of Stanford University was used to determine cytokine and chemokine levels. Human 37-plex or 13-plex kits (Affymetrix Inc.) were used according to the manufacturer's recommendations with the following modifications. Briefly, samples were mixed with antibody-linked polystyrene beads on 96-well filter-bottom plates and incubated at room temperature for 2 h followed by overnight incubation at 4 °C. Plates were vacuum-filtered and washed twice with wash buffer, then incubated with biotinylated detection antibody for 2 h at room temperature. Samples were then filtered and washed twice as above and resuspended in streptavidin-PE. After incubation for 40 min at room temperature, two additional vacuum washes were performed, and samples were resuspended in reading buffer. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument with a lower bound of 100 beads per sample per cytokine. Cytokine and chemokines concentrations in pg/ml were obtained using the standard curve of interest and the MFI of experimental samples. Values obtained from unconjugated effector and unconjugated target cell supernatants were added together and subtracted from values of test samples.

2.8. Cytotoxicity assay

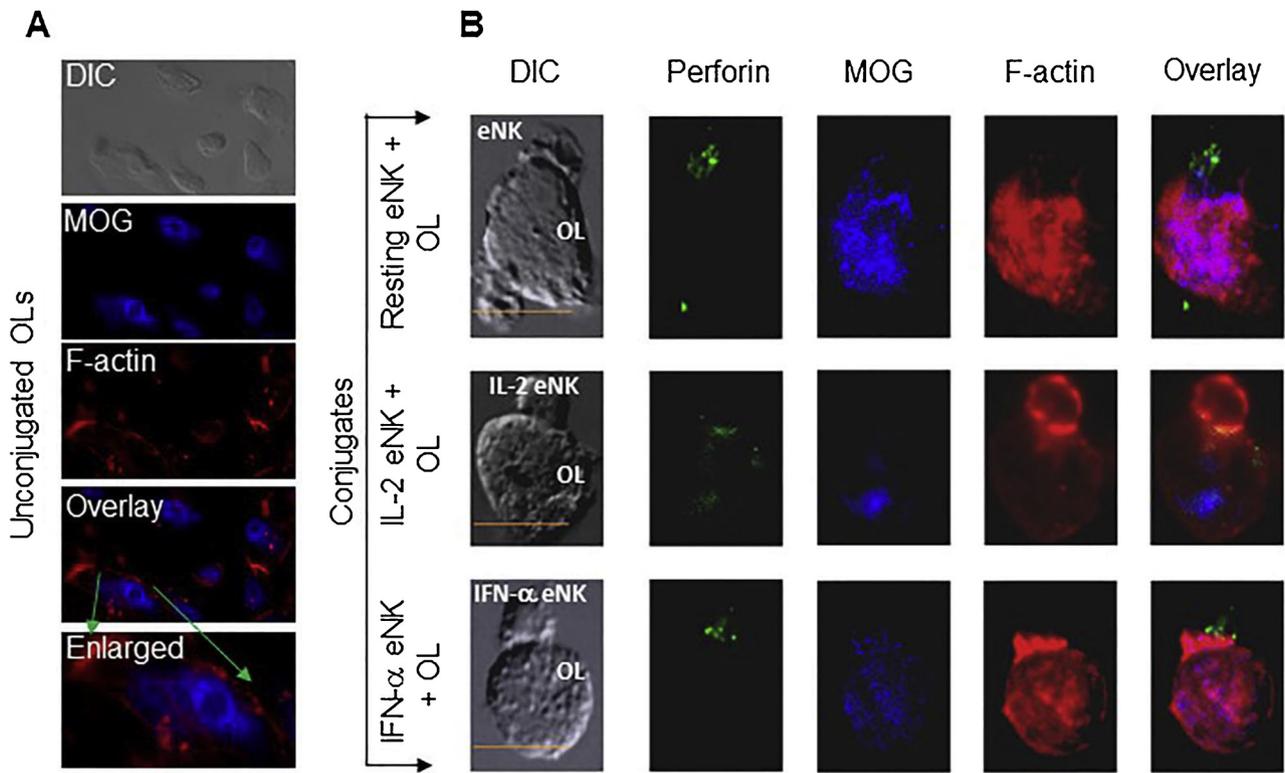
4 h ^{51}Cr -release assays (Banerjee et al., 2007) were performed in v-bottomed 96-well plates. 1×10^6 K562 or OLs, or MO3.13 target cells were resuspended in 100 μl of RPMI with 10% FCS and labeled with 100 microcuri of ^{51}Cr for 1 h, in 200 μl total volume at 37 °C, and in the presence of 5% CO₂. After labeling, cells were washed three times in RPMI with 10% FCS and were used for assays. eNK cells were used as effectors, at different effector-to-target cell ratios (E: T), and against all types of target cells. Percent specific lysis was defined as [(mean of the test wells)-(mean of the spontaneous release wells) / (mean of maximal release wells)-(mean of the spontaneous release wells)] \times 100.

2.9. siRNA transfection

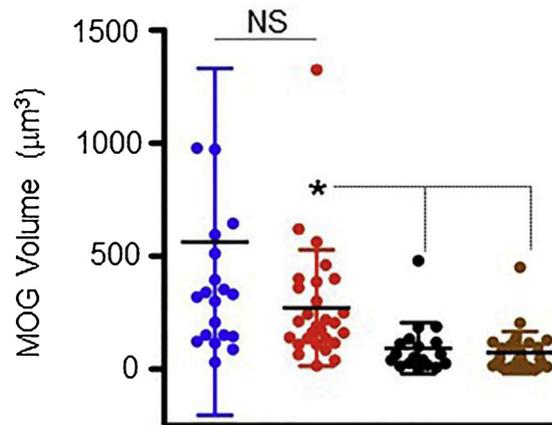
1.5×10^6 WT-KIR2DL4-NK92-GFP cells were nucleofected (Banerjee et al., 2007) with 1000 nM of control or IFN- γ siRNA (Santacruz) in nucleofector solution R (Lonza) using program A024 of the Amaxa Nucleofector II and incubated in serum containing media for 24 h before use.

2.10. Statistical analysis

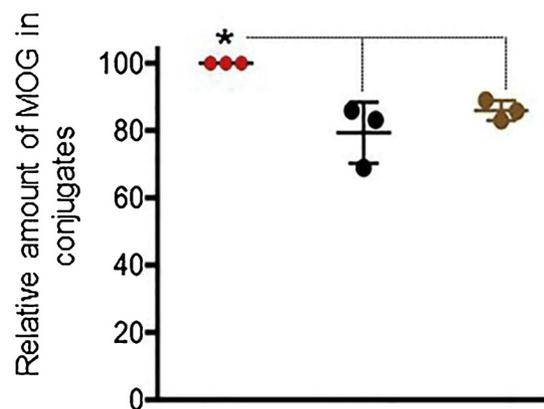
All graphs denote mean values and error bars represent standard deviation (SD). Groups of data were compared using the two-tailed



- C**
- Unconjugated OL
 - Resting eNK+ OL
 - IL-2 eNK+ OL
 - IFN- α eNK+ OL



- D**
- Resting eNK+ MO3.13 cell
 - IL-2 eNK+ MO3.13 cell
 - IFN- α eNK+ MO3.13 cell



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Fig. 2. Activated but not resting eNK cells conjugated to OLs demonstrate MOG-reducing activity. (A) Representative images of an unconjugated OL, and OL in conjugate (B) with resting, IL-2- and IFN- α -treated eNK cells displaying differential interference contrast (DIC) and maximal projections of confocal fluorescence for perforin (green), MOG (blue) and F-actin (phalloidin; red), as well as an overlay of fluorescent channels (enlarged in bottom, A; right, B). Scale bar = 10 μ m. (C) Quantification of MOG volume derived from the 3-dimensional reconstruction of unconjugated OLs and OLs in conjugate with resting (red), IL-2- (black) or IFN- α - (brown) pre-treated eNK cells. Circles represent the volume of MOG from individual OLs. (D) Flow cytometry based assessment of relative MOG content in viable resting NK cell + MO3 cell conjugates (red circles), or that in IL-2 (black) and IFN- α (brown) activated NK cells + MO3 cell conjugates are shown. Each dot represents an experiment. (C, D) Horizontal bars represent the mean and error bars show \pm SD; n = 3 independent experiments. Significant differences between means are noted (* P < 0.05, NS = not significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Student's t test; statistical significance is shown (*, P < 0.05) unless otherwise noted.

2.11. Declaration of approval to study human subjects

All human studies were approved by the institutional review board (IRB) of The Children's Hospital of Philadelphia and Baylor College of Medicine. Written informed consent was obtained from each participant prior to inclusion in the study using an IRB-approved protocol. MS diagnosis and disease severity were determined by a physician certified by the American Board of Psychiatry and Neurology.

3. Results

3.1. Activated but not resting eNK cells mediate cytotoxicity against and form conjugates with oligodendrocytes (OLs)

Human OLs were generated by *ex vivo* differentiation of human oligodendrocyte precursor cells (HOPC). Undifferentiated HOPC, differentiated human OLs and the human oligodendroglial cell line MO3.13 (Buntinx et al., 2003) were evaluated by flow cytometry via intracellular staining (Supplemental Figure S1 A) and Western blotting (Supplemental Figure S1B) for myelin-associated-glycoprotein (MAG) and myelin-oligodendrocyte-glycoprotein (MOG), known phenotypic markers of immature and mature OLs. As expected, MAG expression was restricted mainly to HOPCs (Ma et al., 2009), immature OLs (Poltorak et al., 1987) and undifferentiated MO3.13 cells (Supplemental Figure S1 A), and it was downregulated in fully differentiated MO3.13 cells (Supplemental Figures S1 A, B). In contrast, MOG expression was absent in HOPCs, restricted to cells of oligodendrocyte lineage and further enhanced in fully matured OLs and MO3.13 cells (Supplemental Figure S1 A (Coffey and McDermott, 1997; Solly et al., 1996; Scolding et al., 1989)). We further compared MAG and MOG expression by Western blot analysis and found that in comparison with HOPC, MAG expression was downregulated 4-fold in fully differentiated OLs and 5-fold upon MO3.13 cell differentiation (Supplemental Figure S1B). MOG expression, on the other hand, was upregulated 3-fold in mature OLs, and 1.5-fold upon MO3.13 cell differentiation (Supplemental Figure S1B). These characteristics of *in vitro* differentiated OLs were consistent with the expected phenotype (Coffey and McDermott, 1997; Solly et al., 1996; Ma et al., 2009; Poltorak et al., 1987; Scolding et al., 1989) and flow cytometry-validated preparations of MAG^{low} MOG⁺ cells were used as OLs in subsequent experiments.

IFN- α and IL-2 enhance *ex vivo* natural killer (eNK) cell cytotoxicity against the prototypical erythroleukemic target cell line, K562 (Trinchieri et al., 1984). IL-2, which is elevated in MS patient serum (Gallo et al., 1989), additionally enhances NK cell cytotoxicity against human oligodendrocytes isolated from human brain (Morse et al., 2001). IFN- α -induced cytotoxicity of NK cells is governed by certain activating receptors such as NKG2D and CD161 (Konjevic et al., 2010), and the role of different types of IFNs in MS has been described (Reder and Feng, 2014; Dumitrescu et al., 2018). Thus, we asked whether our *in vitro*-differentiated human OLs were similarly susceptible to cytokine-activated NK cells. Activation of NK cells by either IL-2 or IFN- α enhanced cytotoxicity against OLs (Fig. 1A), and as expected, K562 cells

(data not shown) in 4 h ⁵¹Cr-release assays (Fig. 1A). Human OLs, however, express MHC class-I (Hirayama et al., 1986) and most likely these assays present at least some degree of HLA-mismatch between the NK cells and OLs. We evaluated expression of either class I (A, B, C) or class II (DR, DQ, and DP) molecules on MO3.13 cells and found them to be negative (data not shown). In cytotoxicity assays the MO3.13 cells were killed only by cytokine-activated but not resting primary NK cells (Fig. 1B). This implies that NK cell lytic activity against OLs does not depend upon HLA mismatch.

Because NK cell cytotoxicity represents a contact-dependent phenomenon between effector and target cells, we next wanted to understand the physical contact between NK cells and OLs. We initially asked whether IL-2- and IFN- α -activated NK cells conjugate more efficiently with OLs and MO3.13 cells. Using a flow cytometry-based assay (Fig. 1C), we found that in comparison with resting NK cells, IL-2- (Fig. 1D) and IFN- α - (Fig. 1E) activated NK cells indeed formed more conjugates with OLs and MO3.13 cells. Similarly, slightly more OLs and MO3.1 cells appeared in conjugates (percent of target cells in conjugate) with activated as opposed to resting NK cells and more activated NK cells overall are found in conjugates. Thus cytokines associated with MS pathogenesis can enhance NK cell conjugate formation with and cytotoxicity against OLs and MO3.13 cells.

3.2. Activated but not resting eNK cells in conjugate promote myelin protein loss

Although it is not possible to evaluate clinical demyelination using an *in vitro* system, The loss of MOG from OLs has been associated with demyelination *in vitro* and *in vivo* (Kerlero de Rosbo et al., 1990; Goddard et al., 1999; Reindl et al., 1999; Iglesias et al., 2001; von Budingen et al., 2001; Birgbauer et al., 2004; Zhou et al., 2018). Thus we evaluated whether activated NK cells in conjugates with oligodendrocytes promote MOG loss. We used quantitative 3-dimensional immunofluorescence (Banerjee and Orange, 2010) to measure MOG in OLs either alone or conjugated with eNK cells. The average MOG content of unconjugated OLs (Figs. 2A, C) was comparable to that of OLs conjugated with resting eNK cells (Figs. 2, B, C). When NK cells were pre-activated with IL-2 or IFN- α , however (Figs. 2B, C) the average MOG volume (Fig. 2C) and total amount of MOG per OL (Supplemental Figure S2A) were reduced significantly. There was no alteration in total OL volume (Supplemental Figure S2B) or in OL F-actin content (Supplemental Figure S2C) in conjugates formed with activated NK cells relative to those formed with resting NK cells. This suggests that activated but not resting NK cells specifically reduce MOG content in OLs

Interestingly, perforin was polarized at the interfaces between activated but not resting NK cell + OL conjugates (Fig. 2B) and was positively associated with a decrease in MOG volume. The reduction of MOG protein, however, could arguably be a feature of cell death, especially since these activated NK cells kill OLs and MO3.13 cells (Figs. 1A, B). To address this, we utilized a flow cytometry-based method to measure MOG amounts only in conjugates that were viable (using a viability dye; Supplemental Figure S3A, B). When only the viable cells were considered, the NK cell-mediated reduction in MOG content from MO3.13 cells was greater after NK cell activation (Fig. 2D). Similarly, when compared to resting NK cell + MO3.13 cell

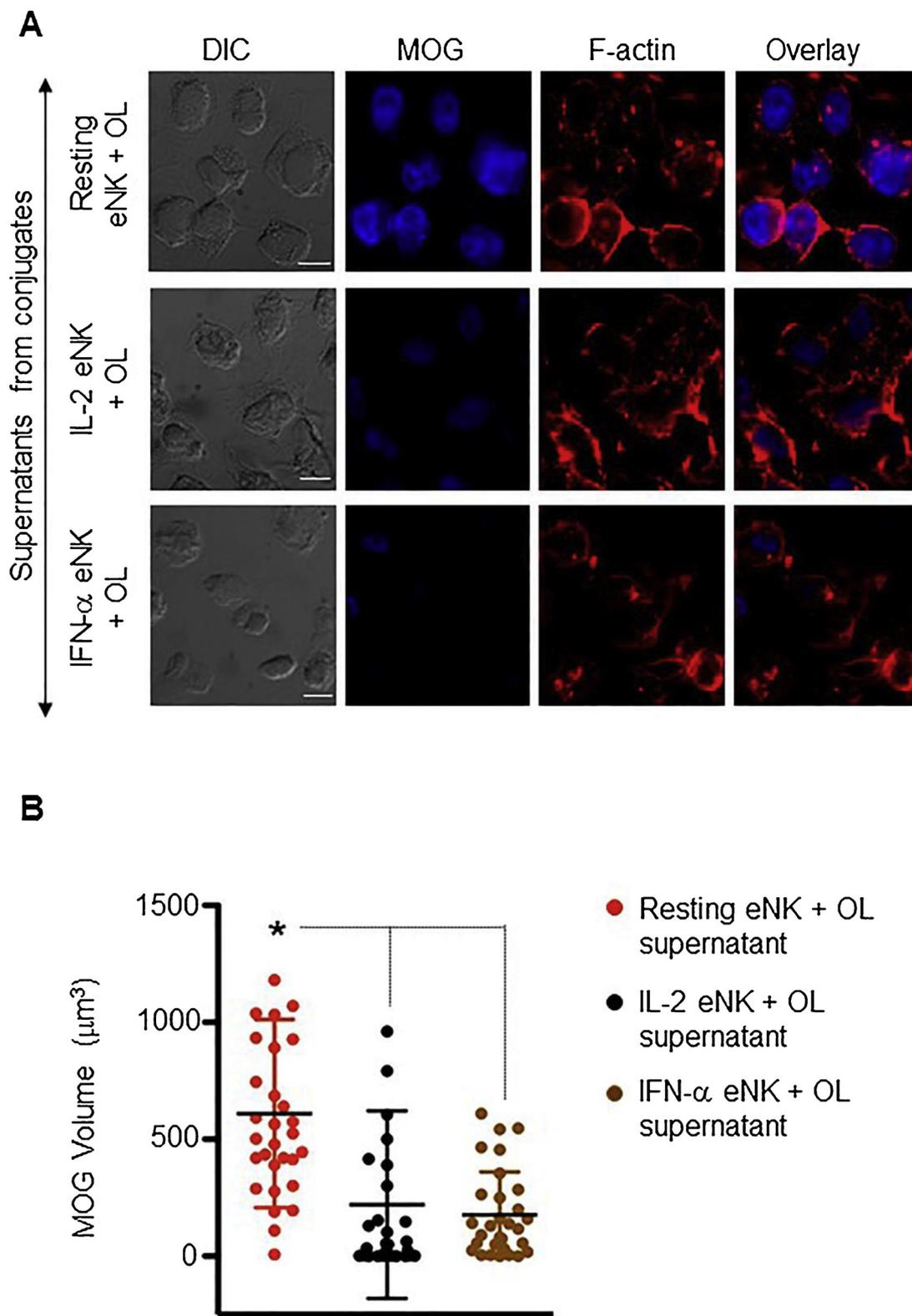
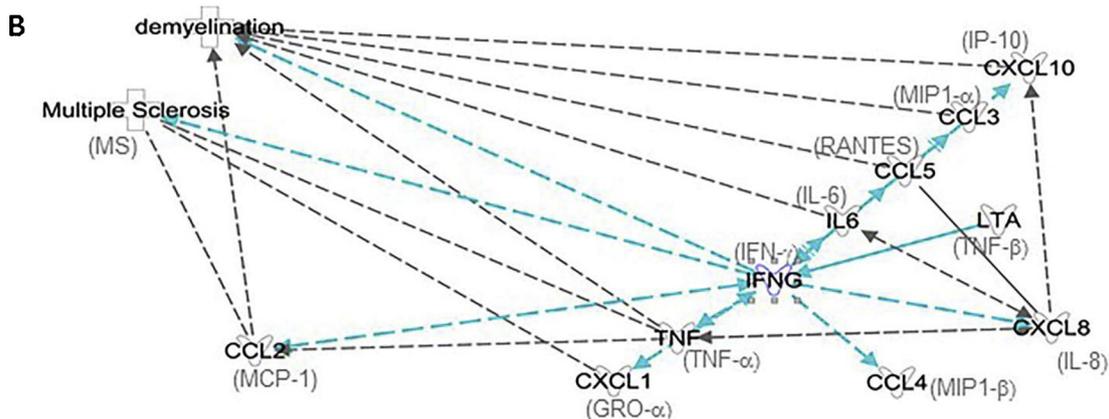
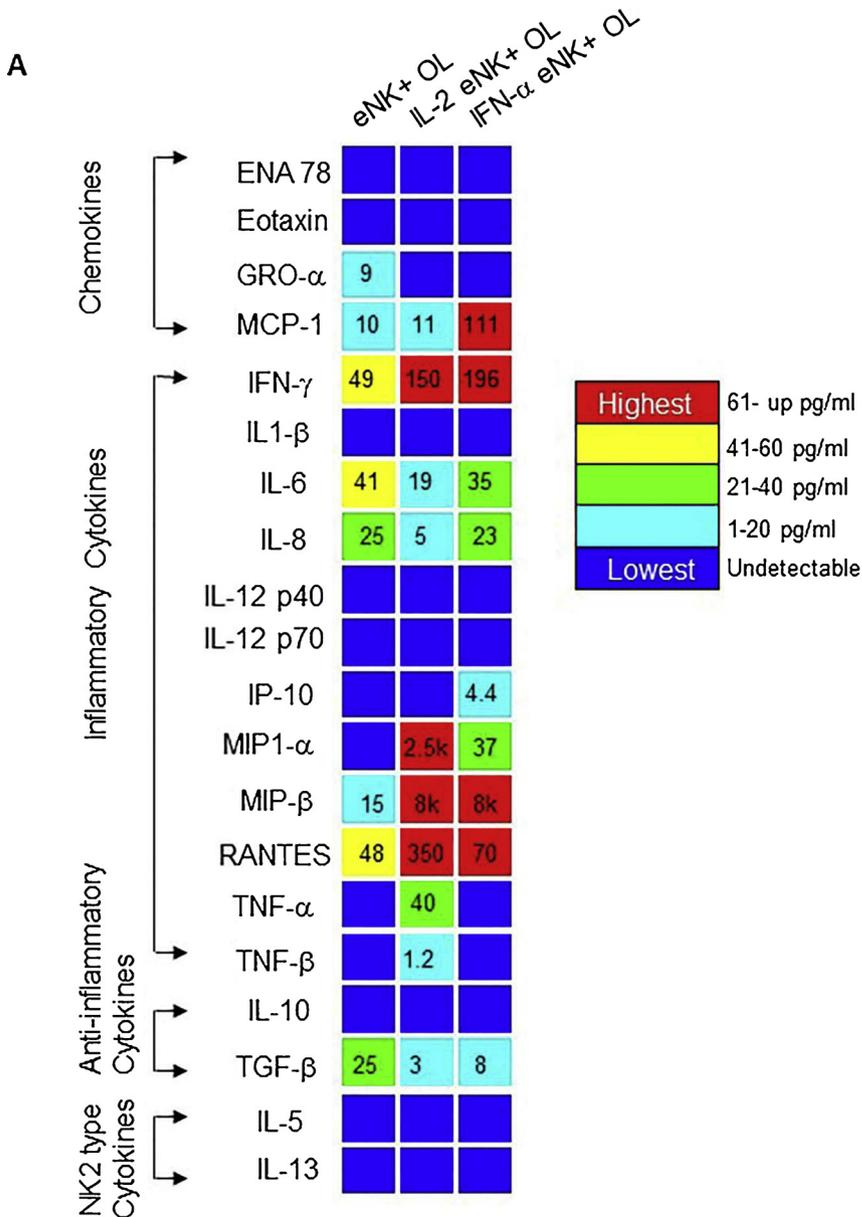


Fig. 3. Supernatants from activated but not resting eNK cell + OL conjugates promote MOG-reducing activity. **(A)** Images of OLS treated with supernatants obtained from conjugates between OLS and resting (top) and IL-2- (middle) or IFN- α - (bottom) activated eNK cells. Representative fields showing DIC (left) and maximal projection confocal fluorescence for MOG, F-actin (phalloidin; red) and an overlay of all fluorescent channels (right). Scale bar = 10 μm . **(B)** Quantification of MOG volume derived from the 3-dimensional reconstruction of OLS treated with supernatants from conjugates between OLS and resting (red) and IL-2- (black) or IFN- α - (brown) treated eNK cells. Circles represent the volume of MOG from individual OLS, horizontal bars represent the mean and error bars show \pm SD; n = 3 independent experiments. Significant differences between means are noted ($*P < 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



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Fig. 4. Cytokine and chemokine profiles of eNK cell + OL conjugate supernatants.

(A) Flow cytometry -based multiplex cytokine and chemokine arrays were performed on supernatants from 16 h conjugates formed between various NK cells and oligodendrocytes. Supernatants from conjugates between: OLs and resting, IL-2- or IFN- α -pre-treated eNK cells were analyzed. Data represent the midpoint of two independent experiments each was corrected with measurements of cytokine and chemokine concentrations from resting, or cytokine activated unconjugated NK cells and unconjugated OLs as baseline controls. Numbers denote concentrations of a particular cytokine or chemokine in pg/ml. The inset color scale denotes comparative levels of each of the chemokines and cytokines measured. (B) Ingenuity Pathway Analysis (IPA) showing role of various cytokines in multiple sclerosis or in demyelinating activity. Effect of IFN- γ (in teal) as a master regulator of various demyelinating cytokines is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

conjugates, myelin-associated glycoprotein (MAG: another surrogate of demyelination (Gendelman et al., 1985; Rahmanzadeh et al., 2018) content was reduced in viable MO3.13 cells conjugated to activated NK cells (Supplemental Figures S3C). These findings suggest that activated NK cells conjugated to oligodendrocytes promote a loss of key myelin proteins.

3.3. Supernatant from conjugates between activated but not resting eNK cells and OLs promotes myelin protein loss

We next asked whether the NK cell activity reducing myelin proteins in OLs could be mediated by soluble factors. Thus, we incubated OLs (in the absence of NK cells) with supernatants obtained from co-cultures of OLs and resting or cytokine activated NK cells. After incubation, we stained supernatant-treated OLs for MOG and F-actin (Fig. 3A). When compared to supernatants from resting NK cells and OLs, supernatants from co-cultures of activated NK cells and OLs substantially reduced both the total volume of MOG (Fig. 3B) and the amount of MOG per OL (Supplemental Figure S2D). Neither activated nor resting co-culture supernatants affected total OL volume (Supplementary Fig. 2E) or F-actin content (Supplementary Fig. 2F), again implying a specific impact of activated eNK + OL supernatants on MOG-reducing activity as opposed to cell integrity. Thus, while NK cells are potentially triggered by intercellular contact, their impact upon OLs can be mediated by soluble factors.

To begin to define which soluble factors contributed to NK cell-mediated MOG reduction in OLs, we compared supernatants from co-cultures of activated (via IL-2 or IFN- α) eNK cells and OLs to those from resting eNK cells and OLs (Fig. 4). Co-cultures from activated NK cells preferentially contained a number of soluble mediators including the NK cell chemoattractants monocyte chemoattractant protein-1 (MCP-1), RANTES, macrophage inflammatory proteins (MIP) such as MIP-1 α , MIP-1 β , as well as IFN- γ . Interestingly, IFN- γ is associated with active demyelinating disease (Panitch et al., 1987) and has been previously characterized as having demyelinating activity (Horwitz et al., 1997; Corbin et al., 1996; Lin et al., 2006; Traugott and Lebon, 1988). TNF- α , which also promotes demyelination (Trenova et al., 2011), was upregulated only by IL-2 pre-activation. Type 2 cytokines produced by NK cells such as IL-5 and IL-13 were undetectable. Ingenuity Pathway Analysis (IPA) software identified each of the upregulated cytokines to be associated with MS or demyelinating activity (Fig. 4B). Thus, activation of eNK cells by IL-2 or IFN- α stimulates NK cells to secrete chemo-attractants and known demyelinating cytokines (Corbin et al., 1996; Horwitz et al., 1997; Lin et al., 2006; Traugott and Lebon, 1988; Panitch et al., 1987; Trenova et al., 2011), upon their co-culture with primary OLs. NK cells are known to produce IFN- γ , one of the demyelinating cytokines, by *in vitro* stimulation with either IFN- α (Matikainen et al., 2001), or IL-2 (Weigent et al., 1983). We, therefore, normalized the data in Fig. 4A to measurements of cytokine and chemokine concentrations from resting, or cytokine-activated unconjugated NK cells and unconjugated OLs as baseline controls. Thus, our data in Fig. 4A only demonstrate the levels of cytokine and chemokine concentrations, which were upregulated in eNK cell-OL culture supernatants. Our results were in agreement with those previously described (Pandya et al., 2011) as we found low (10–20 pg/ml) levels of IL-17 in the supernatants of IL-2 or IFN- α activated unconjugated NK cells. However, the

levels of IL-17 secretion did not increase upon conjugation of resting or cytokine-activated NK cells to OLs. IL-17 had been shown to impair schwann cell -mediated myelination (Stettner et al., 2014), however, as its level did not increase after conjugation of OLs with cytokine activated NK cells in our system we pursued a different mechanism of cytokine activated-NK cell in reducing MOG contents from OLs.

3.4. Oligodendrocytes express HLA-G and can induce NK cell IFN- γ expression and polarization via KIR2DL4

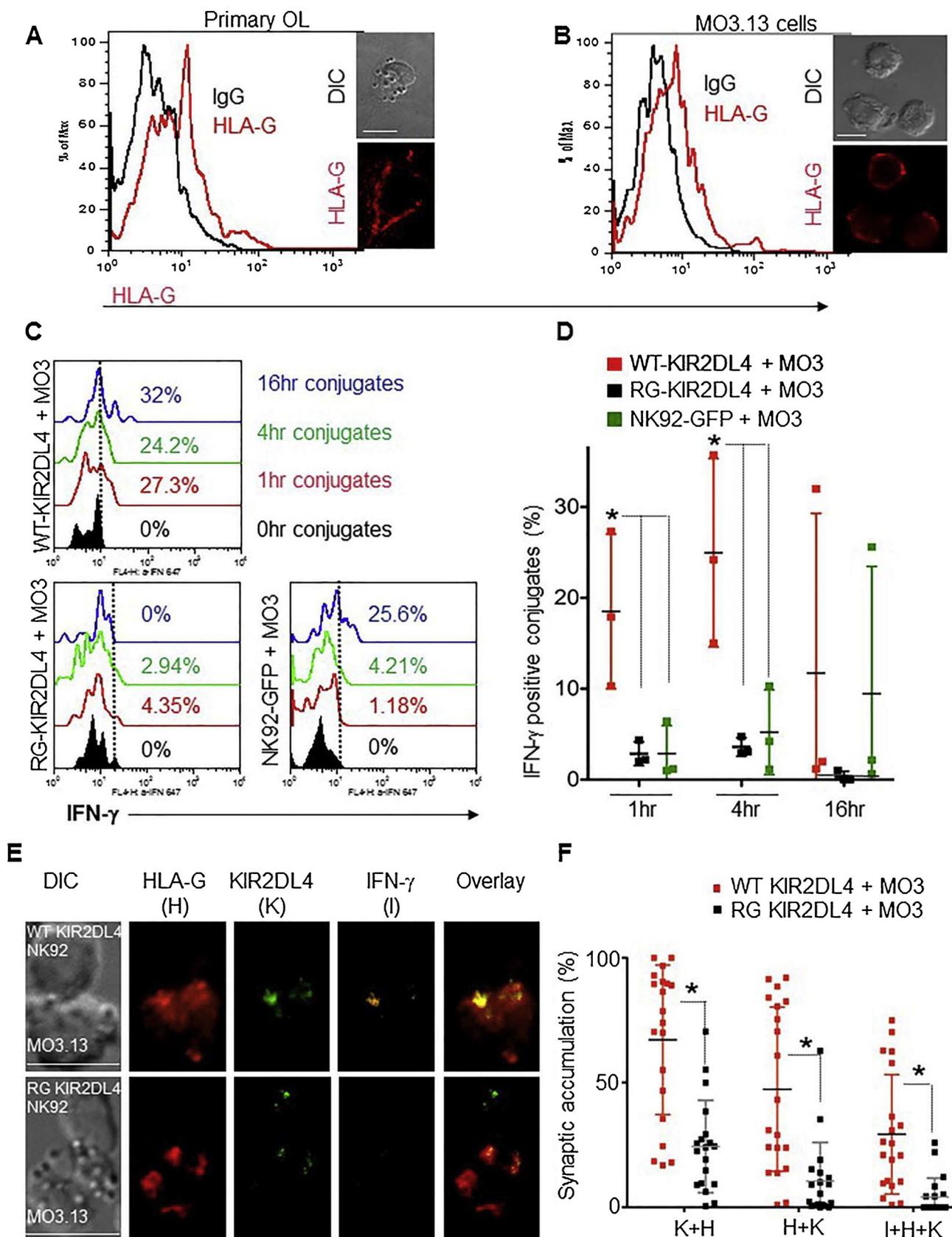
Ligation of the NK cell killer IgG like receptor 2DL4 [KIR2DL4 or CD158d (Kikuchi-Maki et al., 2003)] by human-leukocyte-antigen-G (HLA-G) (Faure and Long, 2002; Kikuchi-Maki et al., 2003; Miah et al., 2008) does not promote NK cell cytotoxicity, but augments secretion of inflammatory cytokines/chemokines including IFN- γ (Kikuchi-Maki et al., 2003; Miah et al., 2008; Rajagopalan et al., 2001). We found IFN- γ and other KIR2DL4-regulated inflammatory mediators in the co-culture supernatants from activated eNK cells and OLs (Fig. 4). Thus, we asked whether a KIR2DL4 ligand (HLA-G) was expressed on OLs, and if so whether it was capable of stimulating NK cell cytokine secretion.

Using flow cytometry and confocal microscopy, we identified HLA-G expression on both primary OLs (Fig. 5A) and MO3.13 cells (Fig. 5B). Therefore we next asked whether KIR2DL4-expressing NK cells secrete IFN- γ when conjugated with MO3.13 cells and whether interfering with KIR2DL4-HLA-G signaling abrogates IFN- γ expression. Because the level of KIR2DL4 expression on primary NK cells varies among individuals, and as much as 25% of the human population does not express KIR2DL4 on their NK cell-surface (Kikuchi-Maki et al., 2003; Goodridge et al., 2003), we stably transduced the human NK cell line NK92 with empty vector (GFP), a vector containing cDNA of wild-type KIR2DL4-GFP (WT-KIR2DL4), or a mutant form of KIR2DL4 (RG) tagged to GFP (RG-KIR2DL4). The latter disrupts association with the Fc ϵ RI- γ and renders the KIR2DL4 receptor unable to trigger IFN- γ production (Miah et al., 2008; Kikuchi-Maki et al., 2005). NK92 cells transduced with WT-KIR2DL4 or RG-KIR2DL4 were sorted for equal GFP expression to ensure equivalent surface KIR2DL4 expression (data not shown). These NK92 cells were then conjugated to MO3.13 cells and assayed for IFN- γ expression by intracellular flow cytometry at various time points (Fig. 5C). NK92 cells transduced with WT-KIR2DL4, but not RG-KIR2DL4, expressed more IFN- γ , relative to the MFI of baseline control obtained at the 0 h time point, after 1 or 4 h conjugation to MO3.13 cells (Fig. 5C, D). Differences after 16 h were not significant.

NK cells are known to polarize IFN- γ to the immunological synapse when in conjugates with susceptible target cells (Barcia et al., 2008). Quantitative confocal fluorescence microscopy demonstrated that conjugation to MO3.13 cells prompts WT-KIR2DL4 cells but not RG-KIR2DL4 cells to polarize IFN- γ at the interface of NK cell and MO3.13 cells within 1 h (Figs. 5E top panel, F) of conjugation. Polarization was contingent upon the expression of WT-KIR2DL4, as the RG-KIR2DL4 consistently resulted in low quantities IFN- γ (Figs. 5C, D) and low polarization of IFN- γ to the interface or synapse (Figs. 5E bottom panel, F).

3.5. KIR2DL4-induced NK cell IFN- γ production promotes OL MOG loss

Since IFN- γ polarizes in NK cells towards OLs dependent upon



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Fig. 5. Oligodendrocytic cells express HLA-G, induce NK cell IFN- γ production and polarization via KIR2DL4. Flow cytometry and confocal fluorescence microscopy showing HLA-G expression on OLs (A) and MO3.13 cells (B) (black line = IgG control, red line = anti-HLA-G). (C) Histograms showing IFN- γ expression in conjugates between DDAOSE-stained MO3.13 cells and WT-KIR2DL4 (top), RG-KIR2DL4 (bottom left) and GFP-expressing (bottom-right) NK92 cells. DDAOSE⁺/GFP⁺ conjugates were evaluated at 0 h (black), 1 h (red), 4 h (green) and 16 h. Dashed lines depict the gate utilized to define percentages of IFN- γ positive conjugates. (D) Plots display points for each independent experiments as shown in C, and horizontal lines display the mean percentages of IFN- γ positive NK92 + MO3.13 cell conjugates at 1 h, 4 h and 16 h of incubation (n = 3 independent repeats, error bars show \pm SD). Percentages of IFN- γ -positive WT-KIR2DL4- (red squares) and RG-KIR2DL4- (black squares) or NK92-GFP- (green squares) cells in conjugates with MO3.13 cells are noted (* P < 0.05). (E) Representative examples of the conjugates formed between a WT-KIR2DL4 (top panel) or RG-KIR2DL4- cell (bottom panel) and a MO3.13 target cell at 1 h. DIC images (left) and confocal fluorescence images demonstrating localization of HLA-G (H, red), KIR2DL4 (K, green), and IFN- γ (I, orange). An overlay of all fluorescent channels (right) is also shown. Scale bar = 10 μ m. (F) Plots display points for each conjugate analyzed and horizontal lines represent the mean percent molecular accumulation of KIR2DL4 with HLA-G (K + H), HLA-G with KIR2DL4 (H + K), and IFN- γ with KIR2DL4 and HLA-G (I + K + H) at the interface of WT-KIR2DL4 (red squares) or RG-KIR2DL4 (black squares) cells and MO3.13 cells. Error bars show \pm SD and significant differences between means are noted (* P < 0.05, n = 3 independent experiments) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

KIR2DL4, we asked if the IFN- γ was promoting MOG loss in OL. We first evaluated the impact of recombinant human IFN- γ on the MOG content of OLs and found that 20 ng/ml, but not 10 ng/ml of IFN- γ for 2.5 hs decreased MOG content. (Fig. 6A, B). In contrast, these concentrations of IFN- γ did not decrease OL cell volume (Supplemental Figure S4 A) or OL F-actin content (Supplemental Figure S4B) suggesting specificity of the IFN- γ -mediated effect.

Since KIR2DL4 ligation induced IFN- γ content and polarization, we next compared the mutant and WT expressing NK92 cells to determine impact upon OL MOG content in conjugated OLs. Using flow cytometry based assays, we found that conjugation with WT-KIR2DL4- but not RG-KIR2DL4-transduced NK92 cells reduced MOG in MO3.13 cells (Figs. 6C, H). To determine if this was mediated via interaction with HLA-G, we pre-incubated MO3.13 cells with a monoclonal anti-HLA-G blocking antibody. HLA-G blocking antibody increased in MOG by 14 +/− 3% (data not shown). To try and more completely block HLA-G a mixture of polyclonal and monoclonal blocking antibodies against HLA-G was added and led to a further increase in OL MOG content of 40 +/− 3% [Fig. 6D, H]. Thus, ligation of KIR2DL4 by HLA-G presumably is required for NK cell-mediated MOG reduction.

Since it is possible that other NK cell secreted contents could promote MOG loss after KIR2DL4 ligation we used siRNA to specifically reduce IFN- γ expression in WT-KIR2DL4 cells (Fig. 6E). When compared with control siRNA-treated WT-KIR2DL4 cells, IFN- γ siRNA resulted in an inability to reduce the amount of MO3.13 cell MOG (Figs. 6F, H). In order to avoid the further manipulation of the NK92 cell line, we next conjugated WT-KIR2DL4 and MO3.13 cells in the presence of a neutralizing antibody against IFN- γ . In these experiments, IFN- γ -neutralizing antibodies, but not control antibodies, abrogated the ability of WT-KIR2DL4 cells to reduce OL MOG content (Figs. 6G, H). Thus, the ability of NK cells to reduce MOG content in OLs requires both IFN- γ and the interaction between WT-KIR2DL4 and its ligand HLA-G (Fig. 6H).

3.6. HLA non-restricted MOG and MAG-reducing activity by NK cells

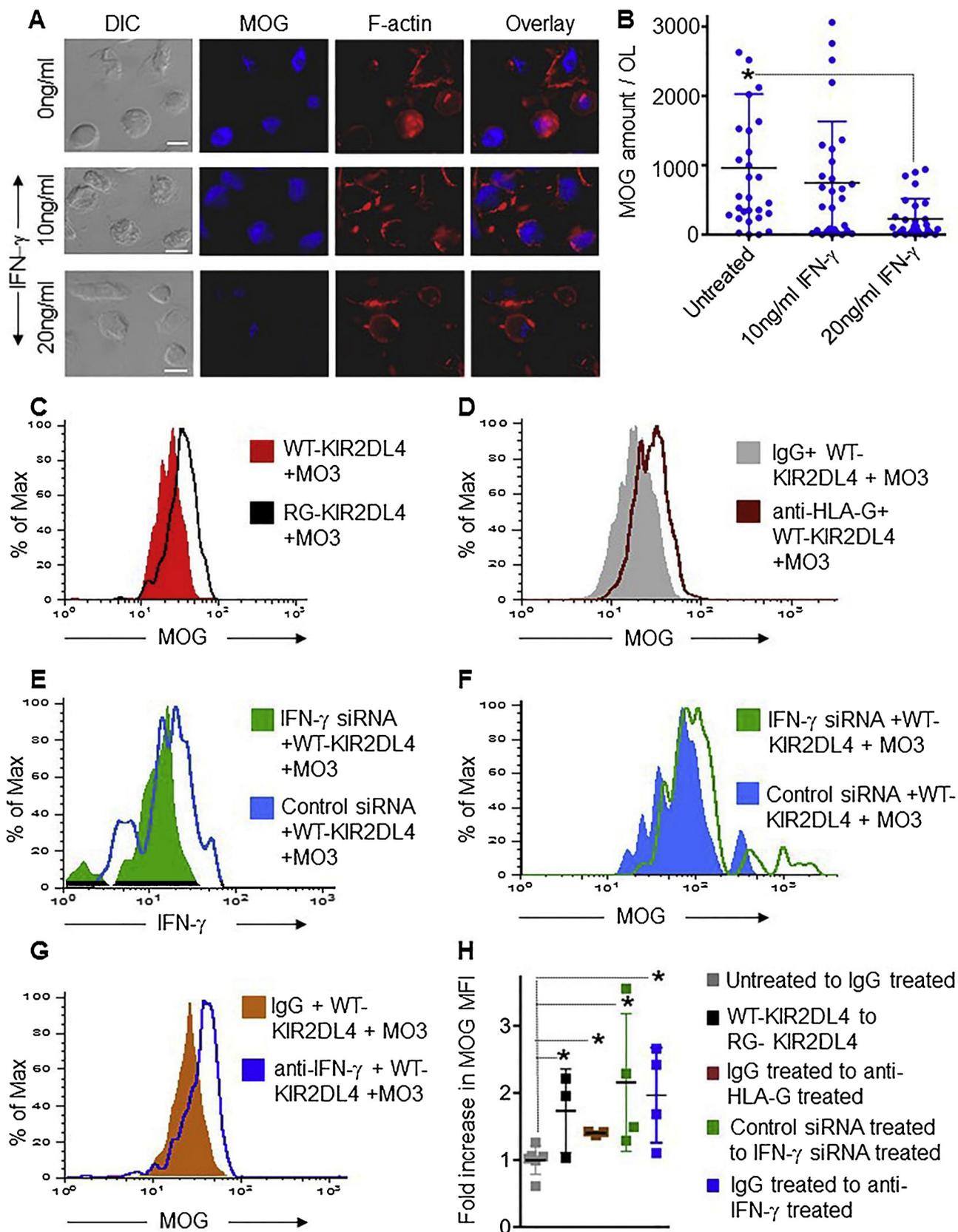
While MO3.13 cells are HLA negative, OLs in human brain are HLA positive (Hirayama et al., 1986; Lv et al., 2014). Thus, to more accurately consider the *in vivo* biology we tested whether the KIR2DL4-dependent MOG-reducing activity identified in NK92 cells was present when the OLs express MHC Class-I. Thus, we stably expressed either HLA-Cw3-GFP or HLA-Cw4-GFP molecules in MO3.13 cells. We obtained HLA Cw3 or Cw4 expression at comparable levels (Fig. 7A) and without altering the expression of HLA-G on them (Fig. 7B). We then checked the expression of KIR2DL1 and KIR2DL4 on various batches of eNK cells and IL-2-activated KIR2DL1 and KIR2DL4-expressing eNK cells. The same set of activated eNK cells were then conjugated with HLA-Cw3 or -Cw4 positive MO3.13 cells to test their myelin protein-reducing activity. Using flow cytometry, and gated on viable eNK cell + MO3 cell conjugates, we found that MO3 cells expressing either HLA-Cw3 or -Cw4 were equally susceptible to MOG- or MAG-reducing activity by activated eNK cells *in vitro* (Fig. 7C).

3.7. MS patient NK cells produce more IFN- γ and have greater MOG-reducing capacity in OL

In an effort to translate our findings more directly to human demyelinating diseases, like multiple sclerosis (MS), we investigated whether *ex vivo* NK cells from MS patients (MS eNK cells) possess enhanced activity against OLs in reducing MOG content, and if so, whether this ability is associated with conjugation-mediated upregulation of IFN- γ . We found (in agreement with others (Vranes et al., 1989)), that PBMCs from MS patients contain significantly lower percentages of CD3⁺ CD56⁺ NK cells compared to control donors (Fig. 7D). MS patient eNK cells, however, synthesized more IFN- γ after 1 h of conjugation with MO3.13 cells than those from control donors (Fig. 7E x axis). This was especially pronounced in patients who were in the midst of disease flares. In parallel experiments, NK cells from the same MS patients were able to reduce a significant amount of intracellular MOG in MO3.13 cells, at 2.5 h, in co-culture (Fig. 7E y axis). The higher production of IFN- γ by MS eNK cells was also significantly (p = 0.041) and negatively correlated (r value of Pearson's correlation coefficient = -0.9915) with reduction in MOG amount from MO3.13 cells in conjugate. The flaring MS patient NK cells that had higher IFN- γ production also had lower MOG content. Interestingly, the KIR2DL4 (CD158d) expression trended towards being higher on MS patient NK cells (and was also represented by a significantly higher percentage of the CD3⁺, CD56⁺ subset) (Fig. 7F). On NK cells, KIR2DL4 expression is mainly restricted to the CD56^{bright} subset (Goodridge et al., 2003). Thus, it was not surprising to find a higher population of CD3⁺, CD56^{bright} cells in MS patients (Fig. 7F). This suggests that KIR2DL4 expression and function may relate to IFN- γ expression which in turn promotes MOG reduction in human OLs. Since KIR2DL4 and HLA-G are expressed only in humans and non-human primates, our findings suggest a human specific role of KIR2DL4 in promoting IFN- γ -dependent myelin protein-reducing activity, which could be relevant to MS pathogenesis and disease progression by contributing to demyelination of neurons.

4. Discussion

To better understand the potential role of human NK cells in MS pathogenesis, we developed an *ex vivo* model of human NK cell-OL interaction. Since the *in vivo* rodent model of experimental autoimmune encephalomyelitis (EAE) is antigen-induced, it only allows to a certain extent of exploration of any potential antigen independent mechanisms of NK cells in pathogenesis of demyelination (Chong et al., 2013; Gao et al., 2016; Hertwig et al., 2016). Many innate immune receptors on human NK cells are also different from those in rodents including the entire KIR family. Thus, early phases of innate immunity-mediated demyelination (Lagumersindez-Denis et al., 2017) are likely to have human-specific characteristics (Hart et al., 2015). To determine if human NK cells in having activity against OLs, we generated human primary OLs by *ex vivo* differentiation of commercially available human oligodendrocytic precursor cells (HOPC) and cultured them with *ex vivo* NK cells. We found that activated, but not resting NK cells had activity



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against OLs. This was independent of MHC-I expression by the OL and thus likely a feature of NK cells being present in the proximity of OLs after having received activation.

Although NK cells do kill OLs (and were previously known to do so),

we wanted to explore some alternative roles of NK cells that could under some circumstances be detrimental to OLs and their critical myelination function. Thus we focused upon an effect NK cells might have on the MOG content of OLs. MOG content has been used as a

Fig. 6. Purified IFN- γ and NK cell produced IFN- γ diminish MOG content of OLs.

(A) DIC (left) and maximal projection confocal fluorescence images of OLs treated with media alone or containing 10 or 20 ng/ml IFN- γ for 2.5 h. MOG fluorescence is shown in blue, F-actin (phalloidin) in red. An overlay of fluorescence channels is shown on the right. Scale bar = 10 μ m. (B) MOG content in untreated or IFN- γ -treated OLs. Each circle represents the amount of MOG in an individual OL. Horizontal bars represent the mean, error bars show \pm SD and significant differences between means are noted (* P < 0.05, n = 3 independent experiments). (C, D) MOG expression measured by flow cytometry at 2.5 h in (C) DDAOSE-labeled MO3.13 cell and WT-KIR2DL4 (red) or RG-KIR2DL4 (black) cell conjugates; or in (D) DDAOSE-labeled MO3.13 cell and WT-KIR2DL4 cell conjugates formed in the presence of control (IgG, grey) or a mixture of anti-HLA-G monoclonal and polyclonal antibodies (brown). (E) IFN- γ and (F) MOG expression measured by flow cytometry at 1 h (E) or 2.5 h (F) in conjugates between DDAOSE-labeled MO3.13 cells and control siRNA (blue) or IFN- γ specific siRNA (green) pre-treated WT-KIR2DL4 cells. (G) MOG expression in conjugates between DDAOSE-labeled MO3.13 cells and WT-KIR2DL4 cells at 2.5 h in the presence of IgG (orange) or anti-IFN- γ (blue) antibody. (H) Horizontal lines on the plot represent the mean fold increase in MOG-MFI over the 3–5 independent experiments performed. Control IgG-treated cells demonstrated no change (gray squares) while up-regulation of MOG in others relative to these was identified as shown. Error bars show \pm SD, and means significantly different from control (grey squares) are noted (* P < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

marker for demyelination *in vitro* (Birgbauer et al., 2004; Kerlero de Rosbo et al., 1990) and *in vivo* (Iglesias et al., 2001; Reindl et al., 1999; von Budingen et al., 2001). Using quantitative three-dimensional image analysis (Birgbauer et al., 2004; Xue et al., 1999), we showed that OLs, exposed to either activated (but not resting) NK cells (Fig. 2) or to supernatant from activated NK cell + OL conjugates (Fig. 3), contained less MOG per OL. These results suggest that activated NK cells harbor some ability to reduce MOG content and that this activity can be mediated by soluble factors. Arguably this decrease in MOG is indirect, reflecting increased killing of OLs by cytokine activated NK cells. However, OL viability, F-actin content and cell volume remained stable throughout our experiments (Supplemental Figures S2-S3 and S4). Others have similarly shown that percent viability of OLs is not affected by demyelination *in vivo* (Goddard et al., 1999) or *in vitro* (Marta et al., 2005). Although we studied MOG reduction at 2.5 hs, it is still possible that these OLs are in the early process of dying, which could represent another mechanism of NK cell in the pathogenesis of MS.

Potential MAG or MOG-reducing activity by NK cells would appear to be contact dependent as so far as certain cytokines such as IFN- γ , and TNF- α , were higher in supernatants of activated NK cell + OL conjugates (Fig. 4) and are known for their direct role in the pathogenesis of MS. The presence of higher amount of RANTES especially in IL-2 activated NK cell-OL supernatant (Fig. 4A) is interesting, as PBMCs from MS patients were also shown to express RANTES (Pittaluga, 2017) and levels of this cytokine in CSF are known to be increased during the onset and progression of MS (Mori et al., 2016). The main source of RANTES in MS pathogenesis, however, is believed to be astrocytes that have been activated by proinflammatory cytokines, such as IFN- γ (Pittaluga, 2017).

The observation that IFN- γ in activated NK cells polarizes towards the OL suggests a possibility for direct cytokine secretion onto the OL, but since we found that IFN- γ in supernatants or when added exogenously (Fig. 6A,B) was effective in reducing MOG content, it is thus hard to say as to whether polarized secretion of cytokine plays any direct physiologic role. IFN- γ polarization at the immunological synapse during the clearance of viral infections from brain cells has been observed in other studies (Barcia et al., 2008). That said, in a complex tissue environment with an active extracellular fluid circulation it is possible that targeted IFN- γ secretion could be relevant to disease progression and local areas of OL impact. Since NK cells can be recruited to the CNS (Huang et al., 2006; Rodriguez-Martin et al., 2015) under certain circumstances, it is possible that NK cells interacting with OLs, promote a local inflammatory cytokine milieu also allowing for other cells, such as through astrocytes secreting RANTES, to contribute to disease progression.

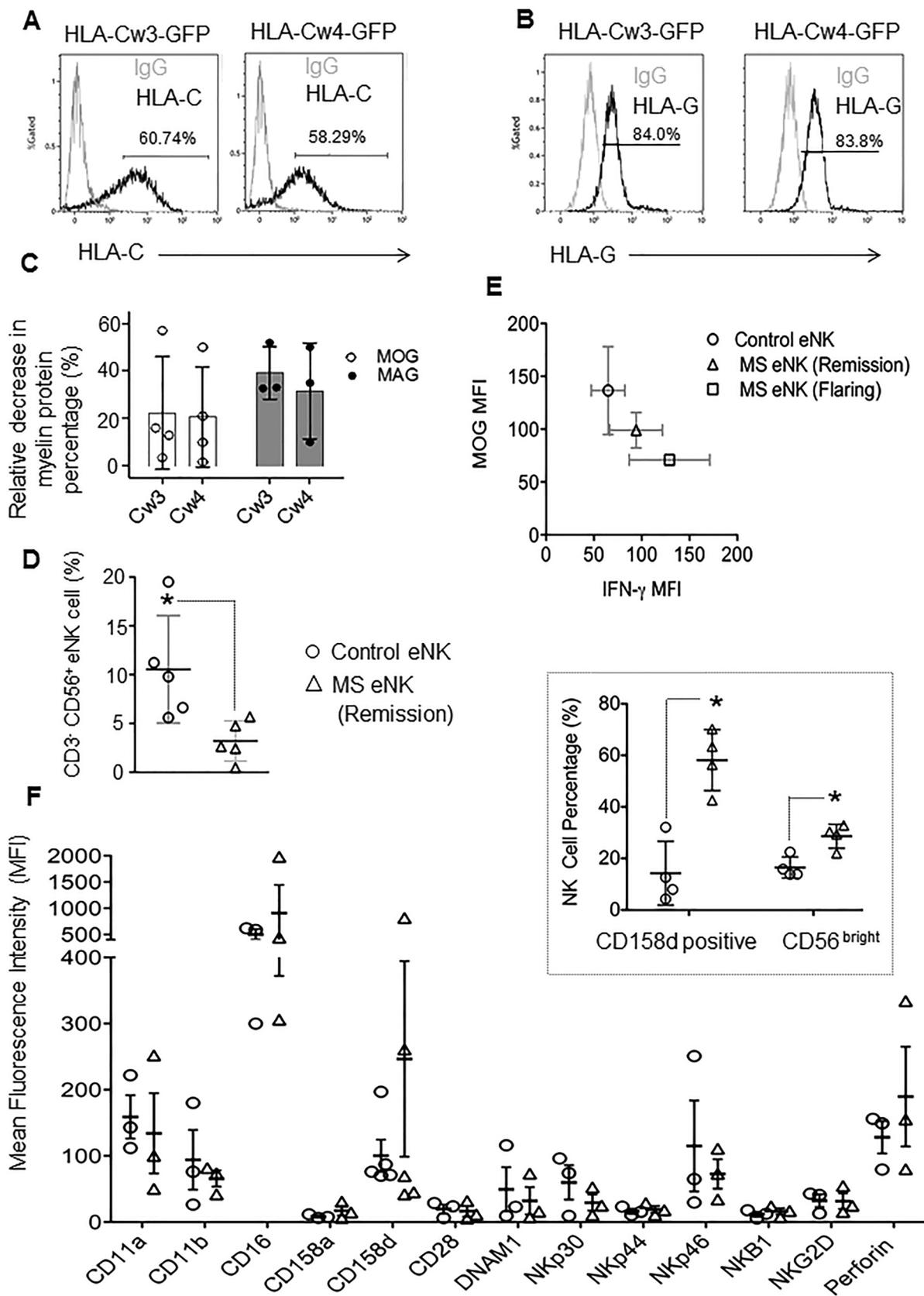
Signaling through the NK cell receptor KIR2DL4 leads to production of IFN- γ and many other cytokines when activated by engagement with the MHC class I-like ligand human-leukocyte-antigen-G (HLA-G) (Faure and Long, 2002; Kikuchi-Maki et al., 2003; Miah et al., 2008). A contribution of HLA-G to MS susceptibility has been suggested (Cree et al., 2010), as immunohistochemistry has identified strong HLA-G expression in CNS tissue from MS patients relative to individuals without CNS

autoimmunity (Wiendl et al., 2005). Thus we confirmed HLA-G expression on the OLs we were using, and further considered the HLA-G/KIR2DL4 interaction in our system (Fig. 5E, F) with regards to IFN- γ -mediated reduction of MOG content in OLs (Fig. 6C-H). As neither KIR2DL4 nor HLA-G is expressed in mice, but many of the KIR ligands are reported to be involved in MS (Kaur et al., 2013; Shahsavari et al., 2016; Hollenbach et al., 2016; Misra et al., 2018) we saw this as a specific opportunity to be approached through our human cell co-culture model. We also found that induction and polarization of IFN- γ by OLs was enabled solely by the expression of WT but not mutant form of KIR2DL4. The use of this modified receptor in NK92 cells was of particular use as they do express a wide variety of NK cell activating receptors including NKG2D, which in activated primary NK cells can induce IFN- γ production and confer cytotoxic activity against OLs (Saikali et al., 2007). However only one of its receptors, the MICB*004 allele, is associated with MS susceptibility (Fernandez-Morera et al., 2008; Babic and Romagnani, 2018). Thus, it is not surprising that NKG2D was expressed at equal levels on eNK cells of healthy donors and MS patients (Fig. 7F), and when expressed on RG-KIR2DL4 (data not shown) it failed to rescue the RG-KIR2DL4-NK92 cells in terms of IFN- γ production even when these cells were incubated with OLs for as long as 16 hs. It would also seem that the role of KIR2DL4 is independent of other MHC Class-I mismatch as MO3.13 cells are class-I negative and our overexpressing class-I alleles (HLA-Cw3 and Cw4) in MO3.13 cells did not have impact on MOG and MAG reduction.

The presence of significantly higher numbers of KIR2DL4-positive NK cells among the NK cell population of MS patients (Fig. 7F insert) is of interest and further supports an important role for KIR2DL4. Further, the higher expression of KIR2DL4 (Fig. 7F) was possibly functionally relevant as there was higher IFN- γ production in these cells upon conjugation to MO3.13 cells (Fig. 7E). It will be of interest to determine if higher expression KIR2DL4 on NK cells and/or higher percentages of KIR2DL4 positive NK cells of MS patients that directly relates to activity against OLs and truly correlates with severity of disease. Should these be the case it will be potentially useful to explore KIR2DL4 expression or the CD3⁻/CD56^{bright} population (Fig. 7F) as a bona fide biomarker for MS disease activity. Finally, it is known that there are significantly fewer circulating NK cells in MS patients (Vranes et al., 1989; Martinez-Rodriguez et al., 2011); a finding we have repeated in a distinct set of MS patients (Fig. 7D). The significance of having reduced NK cells in MS patients and a relatively increased number during disease remission (Montes Diaz et al., 2018) is unclear, but could support a model where peripheral blood NK cells migrate to the CNS (Rodriguez-Martin et al., 2015), interact with OLs, and secrete pro-inflammatory mediators promoting disease progression.

5. Conclusions

Findings in our human cell co-culture system contribute to a model for MS pathogenesis including a role for NK cells. In this model, a precipitating event, such as a viral infection to the central nervous system, would lead to systemic cytokine stimulation of NK cells and



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Fig. 7. HLA- haplotype non-restricted MOG-reducing activity of eNK cells; a role of KIR2DL4 and IFN- γ . (A) Histograms represent expression of HLA-Cw3 (left, black) or HLA-Cw4 (right, black) or (B) HLA-G (black) expression on transfected MO3.13 cells with respective plasmids as mentioned on the top. Numbers on histograms represent percent positive cells over IgG controls (gray). (C) Flow cytometry based myelin protein-reducing activity of IL-2 activated eNK cells in HLA-Cw4 (Cw4) and HLA-Cw3 (Cw3) transduced MO3 cells in conjugate. Relative to unconjugated MO3 cells, decrease in MOG (open circles) and MAG (filled circles) content in MO3.13+ eNK cell conjugates is measured as percent myelin protein-reducing activity at 2.5 h. Each dot represents one experiment; horizontal bars the mean and error bars denote SD. Decrease in MAG and MOG content between the two groups were not significant, $n = 3$ to 4 independent repeats (D) Plots represent the mean percentages of CD3⁻ CD56⁺ NK cells among PBMCs from control donors (circles) and MS patients (triangles). The vertical line demonstrates the mean and error bars show \pm SD, $n = 5$ individual patients and controls. Statistically significant differences in means are noted $*P < 0.05$. (E) IFN- γ (x axis) and MOG (y axis) content in conjugates formed between DDAOSE-labeled MO3.13 cells and CFSE labeled control donor eNK (circle), MS remission patient eNK (triangle) and MS flaring patient eNK cells (square). IFN- γ MFI is significantly and negatively correlated with MOG content ($r = -0.9915$). Data shown were obtained from 3 patients per group and 3 controls. (F) Plots display points for MFI values and the horizontal line represents the mean expression of the various NK cell receptors and perforin on CD3⁻ CD56⁺ NK cells of control donors (circles) or MS patients in remission (triangles). Error bars show \pm SD, $n = 3$ to 5 individuals per group. (Insert) mean percentages of KIR2DL4 (CD158d) positive cells among CD3⁻ CD56⁺ NK cells, or CD3⁻ CD56^{bright} NK cells among PBMCs are depicted from control donors (circles) or MS patients in remission (triangles) with the actual values shown as the individual points and the mean as the horizontal line. Error bars show \pm SD and significant differences between means are noted ($*P < 0.05$, $n = 4$ patients and 4 control donors).

trafficking to the CNS and increased expression of KIR2DL4. KIR2DL4 on activated NK cells could then interact with OL-expressed HLA-G to trigger synthesis of IFN- γ and local secretion. IFN- γ could promote useful functions such as controlling viral replication, but at the cost of reducing MAG and MOG amount in OL, which in turn could affect their myelinating capacity. While MS involves a complex pathogenesis in genetically predisposed individuals a role for innate immunity, and NK cells in particular is conceivable that interfering with these mechanisms may present therapeutic opportunities.

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Conflicts of Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2018.09.027>.

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