Impaired NK-mediated regulation of T-cell activity in multiple sclerosis is reconstituted by IL-2 receptor modulation

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Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) resulting from a breakdown in peripheral immune tolerance. Although a beneficial role of natural killer (NK)-cell immune-regulatory function has been proposed, it still needs to be elucidated whether NK cells are functionally impaired as part of the disease. We observed NK cells in active MS lesions in close proximity to T cells. In accordance with a higher migratory capacity across the blood–brain barrier, $CD56^{bright}$ NK cells represent the major intrathecal NK-cell subset in both MS patients and healthy individuals. Investigating the peripheral blood and cerebrospinal fluid of MS patients treated with natalizumab revealed that transmigration of this subset depends on the $\alpha_4\beta_1$ integrin very late antigen (VLA)-4. Although no MS-related changes in the migratory capacity of NK cells were observed, NK cells derived from patients with MS exhibit a reduced cytolytic activity in response to antigen-activated $CD4^+$ T cells. Defective NK-mediated immune regulation in MS is mainly attributable to a $CD4^+$ T-cell evasion caused by an impaired DNAX accessory molecule (DNAM)-1/CD155 interaction. Both the expression of the activating NK-cell receptor DNAM-1, a genetic alteration consistently found in MS-association studies, and up-regulation of the receptor's ligand CD155 on $CD4^+$ T cells are reduced in MS. Therapeutic immune modulation of IL-2 receptor restores impaired immune regulation in MS by increasing the proportion of CD155-expressing $CD4^+$ T cells and the cytolytic activity of NK cells.

multiple sclerosis | NK cells | daclizumab | DNAM-1 | IL-2 receptor

Multiple sclerosis (MS) is a chronic inflammatory demyelinating autoimmune disease of the central nervous system (CNS) (1) and one of the major causes of neurological disability in young adults (2). MS is considered to be a primarily antigen-driven T cell-mediated disease with a complex genetic background influenced by environmental factors (1, 3) that is caused by an imbalanced immune-regulatory network (4). Among other well-known players of this network such as regulatory T cells and tolerogenic dendritic cells (DCs) (1), natural killer (NK) cells have been recently identified as additional factors in controlling homeostasis of antigen-activated T cells (5, 6).

Originally discovered as antigen receptor-negative innate lymphocytes that play an important role in controlling virus-infected and tumor cells (7), NK cells have also been shown to suppress activated T cells through secretion of anti-inflammatory cytokines and/or cytolytic function (5, 6, 8–12). NK cells lyse target cells in a complex process depending on cell surface expression of certain inhibitory and activating receptors on NK cells and the corresponding ligands on target cells (13). Several activating NK-cell receptors–in particular, NKG2D (CD314) (5, 8, 9, 11, 14), the receptor for MIC-A/B and ULBP1-6, and DNAM-1 (DNAX accessory molecule, CD226) (6, 12, 15), the receptor for Nectin-2 (CD112) and poliovirus receptor (PVR/CD155)–have been proposed to be involved in NK cell-mediated lysis of activated T cells. Of note, polymorphisms in the gene encoding for DNAM-1 have been consistently found in MS-association studies (16–18). Both major NK-cell subsets, namely the $CD56^{bright}CD16^{dim/-}$ and the $CD56^{dim}CD16^+$ subsets (here referred to as $CD56^{bright}$ and $CD56^{dim}$, respectively), seem to be capable of killing activated T cells (19). $CD56^{dim}$ NK cells are the major NK-cell subset in the peripheral blood (PB) (90% of NK cells) and kill

Significance

The importance of natural killer (NK) cells in the control of autoimmunity has recently attracted considerable attention. The current study revealed NK cells as additional players in controlling T-cell activity in CNS autoimmunity. NK-mediated control of T-cell activity in multiple sclerosis (MS) is dysregulated and caused by impaired DNAX accessory molecule-1/CD155 interaction between NK cells and $CD4^+$ T cells. Therapeutic immune modulation of the IL-2 receptor with daclizumab, which has just successfully passed a phase III study in relapsing-remitting MS, not only enhances the cytolytic activity of NK cells but also restores defective NK-mediated immune regulation by increasing the proportion of CD155-expressing $CD4^+$ T cells, thus rendering $CD4^+$ T cells most likely more sensitive to NK-mediated lysis.

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target cells without prior sensitization but only secrete low levels of cytokines (7, 20, 21), whereas CD56^{bright} NK cells are more abundant in secondary lymphoid tissues and inflammatory lesions (75–95% of NK cells), where they produce high amounts of immune-modulating cytokines but acquire cytolytic functions only after prolonged activation (7, 20, 21).

Immune-modulating therapies targeting NK-cell frequencies and cytolytic functions among others such as IFN-β (22–24), glatiramer acetate (25), natalizumab (26, 27), fingolimod (28, 29), and daclizumab (10, 30, 31) point to an immune-protective role of both NK-cell subsets in MS. Daclizumab, a humanized antibody directed against the IL-2 receptor (IL-2R) α-chain (CD25) (reviewed in ref. 4) is a promising MS therapy, which recently showed superior efficacy compared with IFN-β in a phase III study (32). Expansion of peripheral (10, 33) as well as intrathecal (34) CD56^{bright} NK cells under daclizumab treatment correlated positively with therapeutic response (10, 30, 35). Nevertheless, it still remains to be elucidated whether NK-cell immune-regulatory functions are impaired as part of the disease process and whether modulation of the IL-2R with daclizumab restores these deficits or simply boosts NK-cell activity (4). Furthermore, the distribution and function of NK cells in active MS lesions is still poorly understood. Resolving the molecular basis of NK cell-mediated immune control and its potential impairment in MS is important for a better understanding of the role of NK cells in MS pathogenesis and the mechanism of action of NK cell-modulating therapies.

The aim of the current study was to characterize the role of NK cells in the pathogenesis of MS by investigating the presence, distribution, and function of NK cells in three different compartments [CNS, cerebrospinal fluid (CSF), and PB]. Furthermore, a potential deficit in NK-cell immune-regulatory function, its underlying molecular mechanism, and the impact of IL-2R modulation by daclizumab high-yield process (DAC HYP) were explored by studying PB mononuclear cells (PBMCs) derived from clinically stable therapy-naïve MS patients and MS patients receiving daclizumab treatment in comparison with those derived from healthy individuals.

Results

Granzyme K-Expressing NK Cells Are Found in Close Proximity to T Cells in Active MS Lesions. To elucidate distribution and function of NK cells in actively demyelinating MS lesions, immunohistochemistry in CNS tissue specimens was performed (Fig. 1). Actively demyelinating lesion areas were located at the plaque border. These areas were partially demyelinated and infiltrated by numerous macrophages containing myelin-degradation products within their cytoplasm, indicating ongoing demyelination (Fig. 1*A*). Immunohistochemistry for anti-neurofilament revealed relatively preserved axons (Fig. 1*B*). Whereas in the periplaque gray matter, ramified microglial cells were present, the lesion was infiltrated by numerous foamy macrophages (Fig. 1*C*) expressing the activation marker MRP14 (Fig. 1*D*). Within the lesion, multiple vessels with perivascular lymphocyte infiltrates (Fig. 1*E*) consisting of CD3$^+$ T cells (Fig. 1*F*) and few CD20$^+$ B cells (Fig. 1*G*) were found. Strikingly, actively demyelinating MS lesions also contained CD56$^+$ NK cells in close proximity to CD3$^+$ T cells (Fig. 1*H*, yellow arrows). Granzyme K (GrK), which is mainly expressed in the CD56^{bright} NK-cell subset and has been shown to play an important role in NK-cell immune-regulatory function (31), was expressed in lesion-resident NK cells and polarized toward other immune cells (Fig. 1*I*, yellow arrows). GrK expression was not exclusively restricted to NK cells, because we also found GrK-expressing CD56$^-$ cells within the lesions (Fig. 1*I*, white arrows). Interestingly, some of the NK cells found in MS lesions also expressed perforin (Fig. S1), another content of cytotoxic granules involved in NK-cell cytotoxicity. In summary, immune-regulatory GrK-expressing NK cells occurred in active MS lesions, where these cells were found in close proximity to T cells, indicating NK/T-cell interactions in human CNS autoimmunity.

Trafficking Capacity of NK Cells Across the Blood–Brain Barrier Is Not Altered in MS Patients. Expression of GrK in the majority of NK cells located in the CNS (Fig. 1*I*) hints that CNS-resident NK cells are most likely CD56^{bright} NK cells. To investigate whether a higher occurence of CD56^{bright} NK cells is attributable to differences of the two NK-cell subsets in penetrating the BBB (blood–brain barrier), motility (Fig. 2*A* and Movies S1 and S2), adhesion (Fig. 2*B*), and transmigratory capacity (Fig. 2*C* and Fig. S2) of NK cells were investigated using human brain microvascular endothelial cells (HBMECs). Although both NK-cell subsets expressed similar amounts of the leukocyte function-associated antigen 1 (LFA-1) (also known as CD11α/CD18 and $\alpha_L\beta_2$ integrin) (Table 1), CD56^{bright} NK cells exhibited a higher motility (Fig. 2*A*), stronger adhesion (Fig. 2*B*), and migratory capacity (Fig. 2*C*, *Left*) in this in vitro model of the BBB than their CD56^{dim} counterparts. Mimicking inflammatory conditions by stimulating HBMECs with IFN-γ and TNF-α resulted in a stronger adhesion (Fig. 2*B*) and higher migratory capacity (Fig. 2*C*, *Left*) of both NK-cell subsets. Interestingly, NK cells derived from MS patients showed no disease-related alterations in their migratory capacity across the BBB (Fig. 2*C*, *Right*). Taken together, our data revealed that CD56^{bright} NK cells exhibited a higher transmigratory capacity across the BBB and that NK-cell transmigratory features were not impaired in MS.

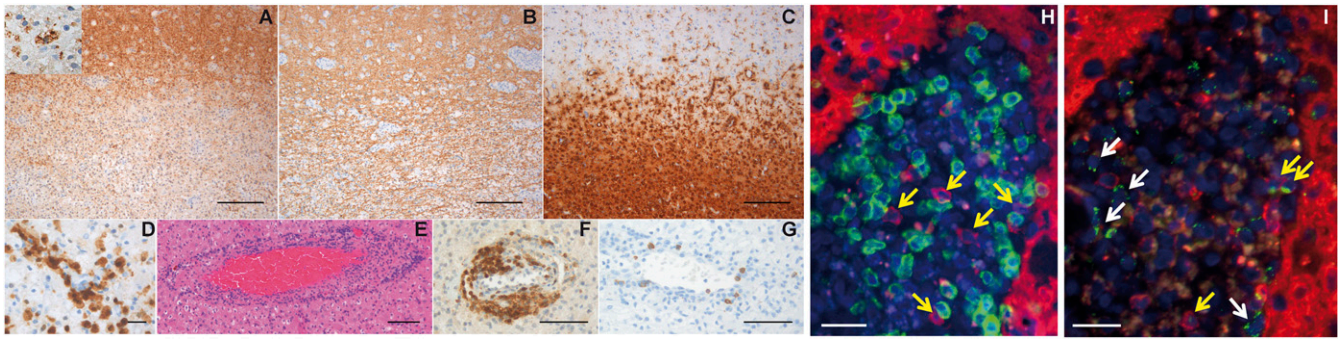

Fig. 1. GrK-expressing NK cells are located in close proximity to T cells in MS lesions. (*A*) Representative immunohistochemical images of a demyelinating subcortical MS lesion characterized by loss of myelin basic protein. (*Inset*) A macrophage with MBP-positive degradation products within the cytoplasm indicating ongoing demyelination (anti-MBP). (*B*) Axons are relatively preserved (anti-neurofilament). (*C*) The lesion is infiltrated by numerous foamy macrophages, whereas in the periplaque gray matter, ramified microglial cells are present (anti-KiM1P). (*D*) The intralesional macrophages express the activation marker MRP14 (anti-MRP14). (*E*) Multiple vessels with perivascular lymphocytic infiltrates within the lesion (H&E staining). (*F* and *G*) The perivascular infiltrates consist of CD3$^+$ T cells (*F*) and few CD20$^+$ B cells (*G*). (*H*) CD56$^+$CD3$^-$ NK cells (anti-CD56, red) are located in close proximity to CD3$^+$CD56$^-$ T cells (anti-CD3, green). (*I*) GrK (anti-GrK, green) is polarized in CD56$^+$CD3$^-$ NK cells (anti-CD56, red). (Scale bars: *A*–*C*, 200 μm; *D*, 25 μm; *E*, 100 μm; *F* and *G*, 75 μm; *H* and *I*, 50 μm.) One representative result of three biopsies from MS patients is shown.

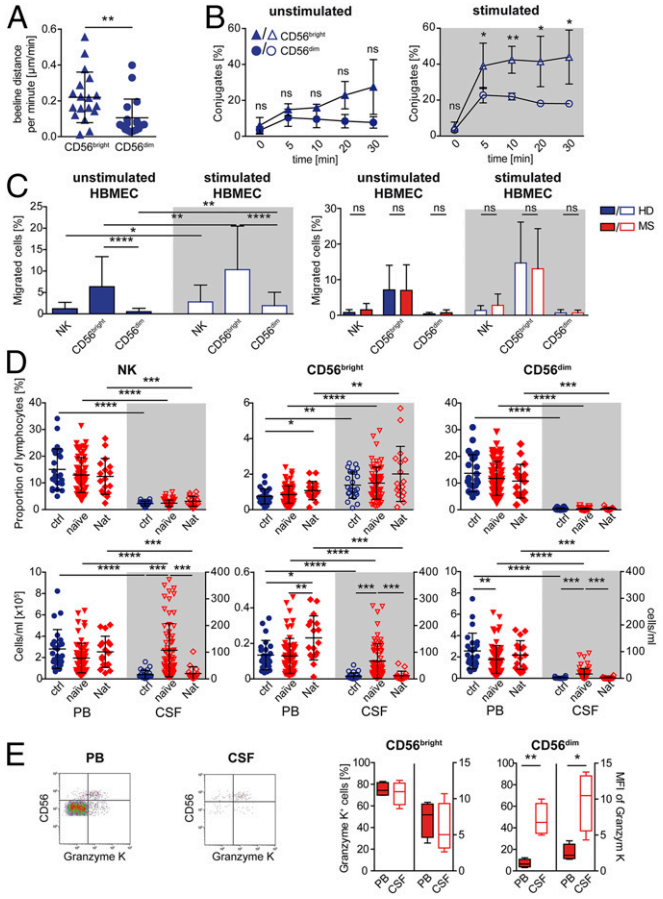

Fig. 2. CD56^{bright} NK cells exhibit a higher migratory capacity in a human BBB model and are the major intrathecal NK-cell subset. (*A*) Covered beeline distance per minute of CD56^{bright} (blue triangles) and CD56^{dim} (blue circles) NK cells (n = 3 HDs) during in vitro incubation with primary HBMECs. Mann–Whitney test was used. (*B*) Conjugate formation of CD56^{bright} (blue triangles) and CD56^{dim} (blue circles) NK cells (n = 4 HDs) with unstimulated and IFN-γ/TNF-α–stimulated HBMECs. Unpaired multiple t test was used. (*C*) Proportions of migrated NK cells and their subpopulations derived from HDs (blue bars) (*Left*: n =21; *Right*: n = 6) or therapy-naïve MS patients (red bars) (n = 5) across unstimulated (filled bars) and stimulated (open bars) HBMECs. Wilcoxon matched-pairs signed rank test (same cell types) and Mann–Whitney test (different cell types/patient groups) were used. (*D*) Proportions (upper row) and total cell numbers (lower row) of NK cells (*Left*), CD56^{bright} (*Center*), and CD56^{dim} (*Right*) subsets in the PB (closed symbols) and CSF (open symbols) of therapy-naïve MS patients (red triangles down) (n = 67) and MS patients treated with natalizumab (Nat) (n = 17) in comparison with HDs (blue circles) (n = 25). Wilcoxon matched-pairs signed rank test (PB vs. CSF) and Kruskal–Wallis test with Dunn's posttest (different patient groups) were used. (*E*) Representative GrK versus CD56 dot plot of CD3$^-$CD56$^+$ NK cells derived from the PB and CSF of therapy-naïve MS patients (*Left*). Proportions and median fluorescence intensity (MFI) of GrK$^+$ CD56^{bright} and CD56^{dim} NK cells in the PB (closed whiskers) and CSF (open whiskers) of therapy-naïve MS patients (*Left*) (n = 4) are displayed. Paired Student's t test was used. Error bars indicate the SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

CSF Distribution Suggests Preferential Very Late Antigen-4–Dependent Trafficking of CD56^{bright} NK Cells into the CNS. Different transmigratory capacities of NK-cell subsets across the BBB model (Fig. 2*C*) were reflected by the composition of intrathecal NK cells (Fig. 2*D*, *Upper*) that is comparable to that in secondary lymphoid tissues (36). Although the majority of NK cells in the PB were CD56^{dim} NK cells, the major NK-cell subset in the CSF of both healthy individuals and MS patients belonged to the CD56^{bright} NK-cell subset (Fig. 2*D*, *Upper*). Proportions of intrathecal NK-cell subsets within the lymphocyte compartment did not differ significantly between MS patients and healthy individuals (Fig. 2*D*, *Upper*), whereas MS patients exhibited significantly enhanced numbers of both CD56^{bright} as well as CD56^{dim} NK-cell subsets (Fig. 2*D*, *Lower*). Differences in the intrathecal NK-cell composition correlated with distinct expression of certain chemokine receptors (Table 1). Whereas CD56^{bright} NK cells were CD62-L$^+$, CX_3CR1^-, CXCR3^{bright}, CCR6$^-$, and CCR7$^+$, CD56^{dim}NK cells were CD62-L$^{+/-}$, CX_3CR1^+, CXCR3^{dim}, CCR6$^-$, and CCR7$^-$ (Table 1). Both NK-cell subsets expressed similar amounts of very late antigen (VLA)-4 [CD49d/CD29, $\alpha_4\beta_1$ integrin (Table 1)], which is involved in transmigration of leukocytes into the CNS (37). Reduced NK-cell frequencies in the CSF of natalizumab-treated (humanized anti-CD49d antibody) patients down to levels of healthy individuals (Fig. 2*D*, *Lower*) suggest that transmigration of NK cells into the CNS is VLA-4–dependent. Of note, NK cells were negative for melanoma cell adhesion molecule (MCAM, CD146) (Table 1), which has recently been identified to be involved in an alternative migratory pathway of Th17 cells across the BBB (38). Finally, a higher proportion of intrathecal CD56^{dim} NK cells expressed GrK in comparison with those of the PB (Fig. 2*E*). In summary, the majority of intrathecal NK cells of both MS patients as well as healthy individuals were GrK-expressing CD56^{bright} NK cells.

Phenotype of Circulating NK Cells Is Altered in MS. To explore potential MS-related changes in circulating NK cells, PBMCs derived from therapy-naïve MS patients with clinically stable disease course were analyzed using multiparameter flow cytometry and compared with healthy individuals (Fig. 3). Interestingly, we observed that NK-cell frequencies, namely the CD56^{dim} NK-cell subset, were significantly reduced in the periphery of MS patients compared with healthy individuals (Fig. 3*A*). Although MS patients showed a reduced cell surface expression of NKp44 (CD336, surrogate marker for NK-cell activation) on a single-cell level, higher proportions NKp44-expressing CD56^{bright} NK cells in MS (Fig. 4*B*) indicated an MS-related activation of this subset. Moreover, lower proportions of IL-2R α-expressing (IL2Rα, CD25) and β-chain–expressing (IL-2Rβ, CD122) (Fig. 3*C*) cells were also found in this subset. Characterization of different activating NK-cell receptors suggested to be involved in NK cell-mediated immune regulation of activated T cells (39) revealed that on the MS cell surface, expression of DNAM-1 was significantly reduced on both NK-cell subsets, whereas no differences in the NK-cell surface expression of NKG2D was observed (Fig. 3*B*). Of note, NK cells from MS patients also expressed lower amounts of 2B4 (CD244), an activating coreceptor that either synergizes with DNAM-1 or NKG2D to trigger NK-cell cytotoxicity (40, 41). In contrast to MS-related alterations of certain

Table 1. Expression of adhesion molecules and chemokine receptors on NK-cell subsets

Surface molecule	Mean percentage of positive cells ± SD, % (MFI)	
	CD56^{bright}	CD56^{dim}
CD11a	99.95 ± 0.15 (14)	99.99 ± 0.02 (14)
CD29	100 ± 0.00 (3)	99.97 ± 0.04 (2)
CD49d	97.06 ± 2.75 (6)	95.90 ± 3.91 (5)
CD146	0.00 ± 0.00 (ND)	0.01 ± 0.02 (ND)
CD62-L	80.66 ± 6.48 (19)	26.71 ± 8.32 (8)
CX_3CR1	15.56 ± 10.04 (3)	95.57 ± 7.41 (6)
CXCR3	74.70 ± 28.38 (29)	10.50 ± 8.96 (15)
CCR6	4.89 ± 3.47 (ND)	1.75 ± 1.91 (ND)
CCR7	57.44 ± 23.59 (2)	5.24 ± 6.53 (ND)

PBMCs of healthy individuals (n = 10) were stained with fluorochrome-conjugated lineage-specific antibodies (CD3, CD56, and CD16), and expression of various adhesion molecules and chemokine receptors was determined on CD56^{bright} and CD56^{dim} NK cells subsets by flow cytometry using the respective fluorochrome-conjugated antibodies. Mean percentage of positive cells ± SD are displayed; MFIs are given in parentheses. ND, not defined.

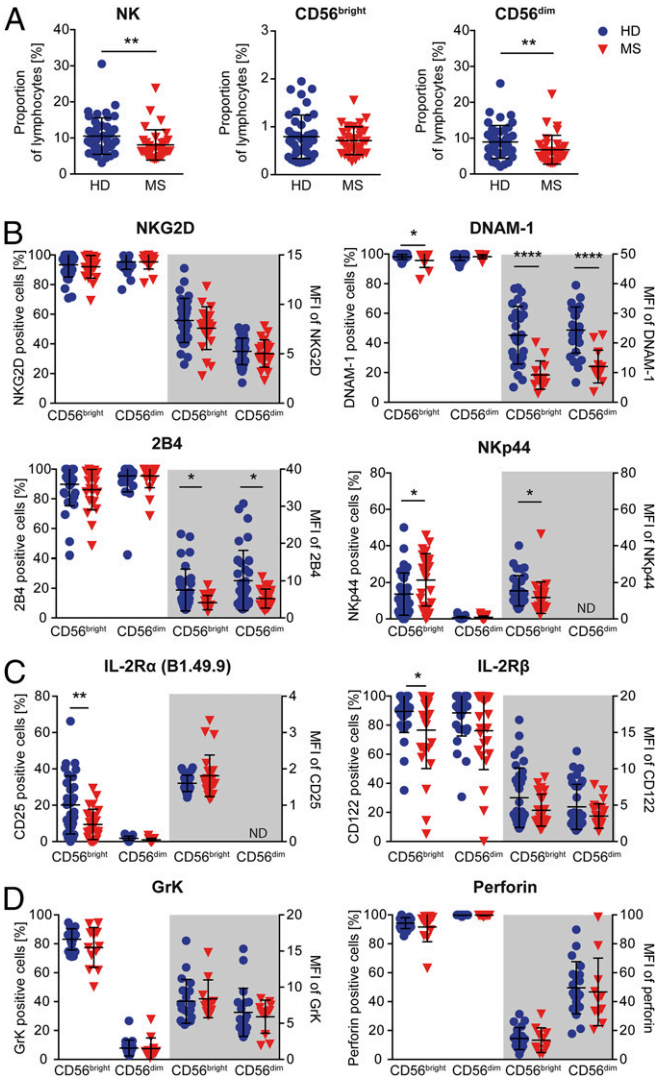

Fig. 3. MS-related changes in the peripheral NK cell compartment. PBMCs derived from healthy individuals (blue circles) and therapy-naïve MS patients characterized by clinically stable disease course (red triangles down) were stained with fluorochrome-conjugated lineage-specific antibodies (CD3, CD56, and CD16) (*A*) and antibodies directed against activating NK-cell receptors [NKG2D (HD, $n = 32$; MS, $n = 25$), DNAM-1 (HD, $n = 28$; MS, $n = 13$), 2B4 (HD, $n = 33$; MS, $n = 25$), and NKp44 (HD, $n = 33$; MS, $n = 25$)] (*B*); IL-2Rα (CD25, clone B1.49.9) and IL-2Rβ (CD122) chain (HD, $n = 33$; MS, $n = 25$) (*C*); and the content of cytolytic granules GrK (HD, $n = 19$; MS, $n = 15$) and perforin (HD, $n = 20$; MS, $n = 15$) (*D*) and analyzed by flow cytometry. (*A*) Proportions of $CD3^-CD56^+$ NK cells and NK-cell subpopulations ($CD56^{bright}$ and $CD56^{dim}$) in the PB of HDs ($n = 43$) and therapy-naïve MS patients ($n = 33$). (*B–D*) Percentages (left side) and MFIs (right side) of cell surface receptor-positive (*B* and *C*) and GrK- or perforin-positive (*D*) $CD56^{bright}$ and $CD56^{dim}$ NK cells. Error bars indicate the SD. *P* values were calculated with Mann–Whitney test. $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$.

activating NK-cell receptors, the content of the cytolytic granules including GrK and perforin was comparable between MS patients and healthy individuals (Fig. 3*D*). Overall, our data showed that MS has an impact on the phenotype of circulating NK cells.

NK-Cell Cytolytic Activity Toward Antigen-Activated $CD4^+$ T Cells Is Impaired in MS. To investigate whether the MS-related reduced cell surface expression of the activating NK-cell receptors DNAM-1 and 2B4 (Fig. 3*B*) had any impact on NK-cell cytolytic function, the release of cytolytic granules (degranulation) in response to both MHC class I-deficient target cells as well as MHC class I-expressing $CD4^+$ T cells was investigated (Fig. 4*A*). Despite reduced cell surface expression of DNAM-1 and 2B4, degranulation of low-dose IL-2–activated NK cells (closed symbols) in response to MHC class I-deficient K562 (Fig. 4*B*, *Left*) as well as 721.221

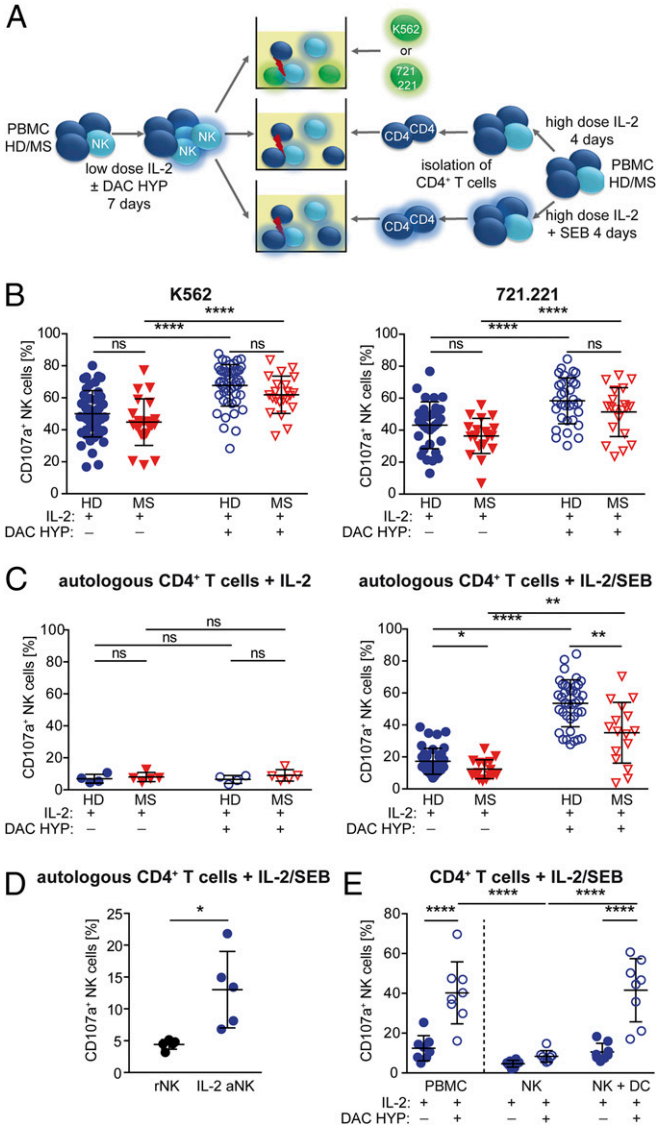

Fig. 4. Impact of IL-2R modulation on NK-cell cytolytic activity. (*A*) Experimental setup: $CD4^+$ T cells were isolated from PBMCs of HDs or therapy-naïve stable MS patients stimulated for 4 d with IL-2 with or without SEB. For NK-cell activation, PBMCs of HDs (blue circles) or MS patients (red triangles down) were stimulated for 7 d with IL-2 with or without DAC HYP. Release of cytolytic granules (degranulation) of IL-2–stimulated (closed symbols) or IL-2/DAC HYP-stimulated (open symbols) $CD3^-CD56^+$ NK cells in response to K562 (HD, $n = 24$; MS, $n = 22$) (*B*, *Left*), 721.221 (HD, $n = 32$; MS, $n = 20$) (*B*, *Right*), and autologous IL-2–stimulated (HD, $n = 4$; MS, $n = 5$) (*C*, *Left*) or IL-2/SEB-stimulated (HD, $n = 38$; MS, $n = 16$) (*C*, *Right*) $CD4^+$ target cells was analyzed. (*D*) Degranulation of resting (black circles) and IL-2–activated (blue circles) NK cells ($n = 5$ HDs) in response to autologous IL-2/SEB-stimulated $CD4^+$ T cells. (*E*) PBMCs, NK cells, and NK cells plus DCs ($n = 8$ HDs) were stimulated for 7 d with IL-2 with or without DAC HYP, and degranulation in response to IL-2/SEB-activated $CD4^+$ target cells was determined. D'Agostino–Pearson omnibus normality test was performed to test for Gaussian distribution. Depending on the result, unpaired Student's *t* test or Mann–Whitney test was used to compare means between two independent groups, whereas paired Student's *t* test or Wilcoxon matched-pairs signed rank test was used for different treatments within the same patient group. $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$.

(Fig. 4*B*, *Right*) target cells was not diminished in MS patients. In contrast, NK cells derived from MS patients exhibited a significantly reduced degranulation in response to MHC I-expressing antigen-activated CD4$^+$ T cells (Fig. 4*C*, *Right*). On the one hand, antigen activation of CD4$^+$ T cells was a prerequisite for NK-cell activation, because NK-cell cytolytic function was only induced when CD4$^+$ T cells were stimulated with IL-2 in combination with staphylococcal enterotoxin B (SEB) (Fig. 4*C*, *Right*) but not with IL-2 alone (Fig. 4*C*, *Left*). On the other hand, IL-2 activation of NK cells was also a prerequisite, because freshly isolated resting NK cells did not respond to IL-2/SEB-activated T cells (Fig. 4*D*). Taken together, our data demonstrated that the cytolytic activity of NK cells in response to autologous antigen-activated CD4$^+$ T cells seemed to be impaired as one feature of MS (Fig. 4*C*).

IL-2R Modulation Enhances Degranulation of NK Cells in a Process Depending on the Presence of Dendritic Cells. To investigate the impact of IL-2R modulation on NK-cell cytolytic activity, release of cytolytic granules in response to K562, 721.221 (Fig. 4*B*), or antigen-activated CD4$^+$ T target cells (Fig. 4*C*, *Right*) was determined in PBMCs stimulated with either low-dose IL-2 alone or IL-2 in the presence of DAC HYP (Fig. 4*A*). Neutralization of the IL-2Rα chain resulted in a significantly increased cytolytic activity of NK cells in response to both MHC class I-deficient target cells (Fig. 4*B*) as well as MHC class I-expressing antigen-activated CD4$^+$ T cells (Fig. 4*C*, *Right*). However, NK cells derived from MS patients still exhibited a significantly reduced cytolytic activity toward antigen-activated CD4$^+$ T cells (Fig. 4*C*, *Right*). Interestingly,

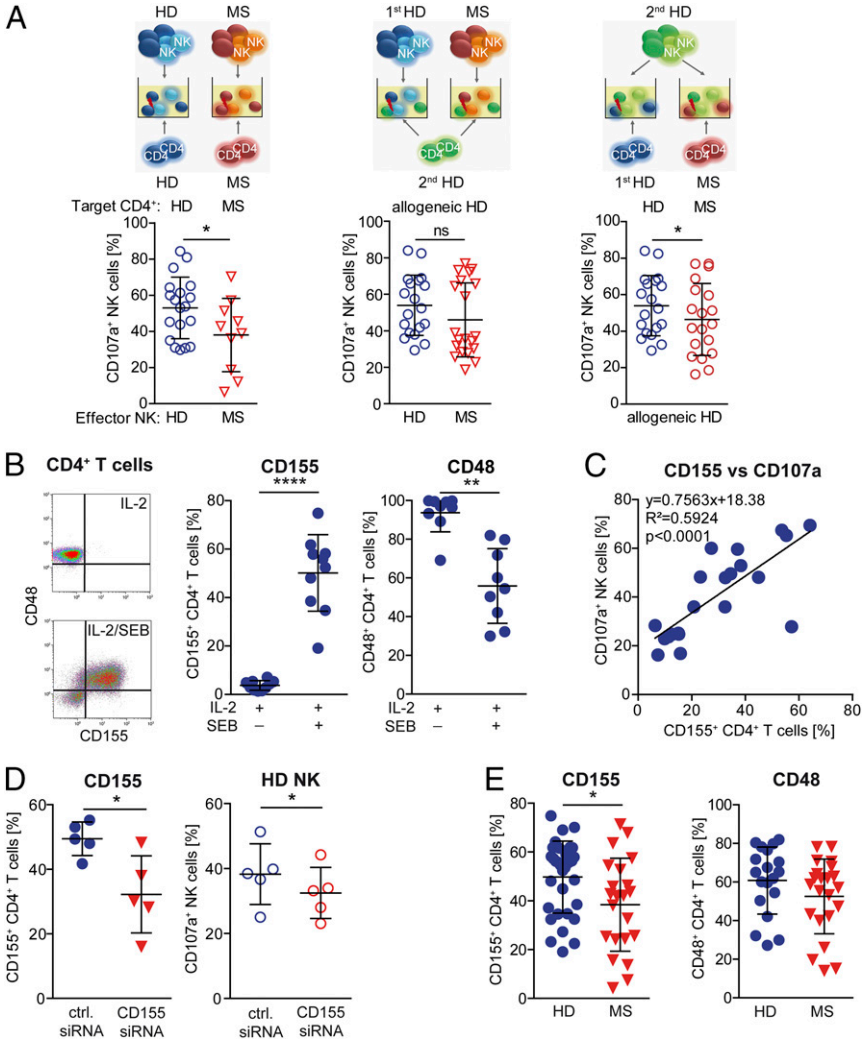

Fig. 5. Impaired DNAM-1/CD-155 interactions result in lower cytolytic activity of NK cells in MS. (*A*) Illustration of crisscross experiments to investigate defective NK-mediated immune regulation in MS (upper row). (*A*, *Left* and *Center*) Degranulation of IL-2/DAC HYP-stimulated NK cells derived from HDs (blue circles; $n = 19$) or MS patients (red triangles; $n = 10$) in response to IL-2/SEB–activated CD4$^+$ T cells derived from the same donor (syngenic setup) (*Left*) or an independent HD (allogeneic setup) (*Center*). (*A*, *Right*) Degranulation of IL-2/DAC HYP-stimulated NK cells of HDs in response to allogeneic IL-2/SEB-activated CD4$^+$ T cells derived either from HDs (blue circles) or MS patients (red circles). Unpaired Student's *t* test (*Left*), Mann–Whitney test (*Center*), and paired Student's *t* test (*Right*) were used. (*B*, *Left*) Dot plot showing CD155 and CD48 expression on CD4$^+$ T cells stimulated for 4 d with IL-2 and IL-2/SEB. (*B*, *Center* and *Right*) Proportions of CD155-expressing ($n = 10$) (*Center*) and CD48-expressing ($n = 9$) (*Right*) IL-2 with or without SEB-activated CD4$^+$ T cells derived from HDs. Wilcoxon matched-pairs signed rank test was used. (*C*) Proportions of CD107a$^+$ NK cells after coincubation with syngenic CD4$^+$ T cells stimulated for 2 and 4 d with IL-2/SEB displayed against proportions of the corresponding CD155$^+$ CD4$^+$ T cells. Correlation was analyzed by Pearson test. (*D*, *Left*) Proportions of CD155-expressing CD4$^+$ T cells transfected with control (blue circles) or CD155-specific siRNA (red triangles) during stimulation with IL-2/SEB. (*D*, *Right*) Degranulation of syngenic NK cells in response to control (blue circles) or CD155-specific siRNA-treated (red circles) activated CD4$^+$ target cells. Paired Student's *t* test. (*E*) Expression of CD155 ($n = 33$HD/22MS) (*Left*) and CD48 ($n = 19$ HDs/22 MS patients) (*Right*) on CD4$^+$ T cells derived from HDs (blue circles) or MS patients (red triangles) following 4-d stimulation with IL-2/SEB. Unpaired Student's *t* test was used. Error bars indicate the SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$.

increased degranulation of NK cells in response to antigen-activated CD4$^+$ T cells was lost when NK cells were purified before stimulation with IL-2/DAC HYP but restored by the addition of myeloid DCs (mDCs) (Fig. 4*E*). In summary, IL-2R modulation boosts NK-cell cytolytic activity in a DC-mediated process.

Impaired DNAM-1/CD155 Interactions in MS Result in Decreased NK-Cell Cytolytic Activity. We next explored the cellular and molecular origin of the regulatory disturbance by performing crisscross experiments (Fig. 5*A*). Therefore, degranulation of NK cells derived from therapy-naïve MS patients in response to autologous CD4$^+$ T cells (Fig. 5*A*, *Left*) or allogeneic CD4$^+$ T cells derived from independent healthy individuals (Fig. 5*A*, *Center*) was compared with that of healthy individuals. In a third setup, the cytolytic activity of NK cells derived from healthy individuals was tested in response to allogeneic CD4$^+$ T cells derived from independent healthy individuals or therapy-naïve MS patients (Fig. 5*A*, *Right*). Crisscross experiments revealed that NK cells from MS patients either exhibited a higher or lower cytolytic activity toward allogeneic healthy donor (HD) CD4$^+$ T cells than those derived from healthy individuals (Fig. 5*A*, *Center*). On the other hand, CD4$^+$ T cells derived from MS patients induced significantly less cytolytic activity in NK cells of healthy individuals than those derived from HDs (Fig. 5*A*, *Right*), although to a lower degree than observed in the syngenic setup (Fig. 5*A*, *Left*). These data indicated that CD4$^+$ T cells of MS patients are at least partially responsible for the defective immune-regulatory function. To understand the mechanism of action behind this MS-related CD4$^+$ T-cell evasion, we further investigated which NK-cell receptor–ligand interaction was responsible for T cell-dependent induction of NK cell-mediated cytolysis. Therefore, we compared the expression of the respective ligands for the activating NK-cell receptors NKG2D, DNAM-1, 2B4, NKp30, NKp44, and NKp46 on CD4$^+$ T cells stimulated with IL-2 alone or in combination with SEB (Fig. S3*A*). IL-2–activated CD4$^+$ T cells, which failed to trigger NK-cell degranulation (Fig. 4*C*, *Left*), mainly expressed the 2B4 ligand CD48 on their cell surface (Fig. 5*B* and Fig. S3*A*). Stimulation of CD4$^+$ T cells with IL-2 in combination with SEB that had been shown to trigger cytolytic activity of NK cells (Fig. 4*C*, *Right*) resulted in a selective up-regulation of the DNAM-1 ligand CD155 (Fig. 5*B*) on CD4$^+$ T cells, but none of the other ligands (Fig. S3*A*), suggesting that CD155/CD48 coexpression on antigen-activated CD4$^+$ T cells might be responsible for induction of cytolytic activity in NK cells. Of note, CD155 expression was observed on all CD4$^+$ T-cell subsets with the strongest up-regulation on central memory T cells (Fig. S3*B*). Our hypothesis that coexpression of CD155 is required for triggering cytolytic activity was further supported by the observed positive correlation between CD155 expression levels on CD4$^+$ T cells and the degree of degranulation of NK cells (Fig. 5*C*). Strikingly, interference with up-regulation of CD155 on the cell surface of antigen-activated CD4$^+$ T cells using siRNA (Fig. 5*D*, *Left*) significantly impaired the cytolytic activity of NK cells (Fig. 5*D*, *Right*), indicating that CD155 is indeed responsible for CD4$^+$ T cell-mediated induction of NK-cell cytolytic activity. In accordance with the MS-related decreased cytolytic activity of NK cells in response to antigen-activated CD4$^+$ T cells (Fig. 4*C*), significantly less CD4$^+$ T cells of MS patients up-regulated CD155 on their cell surface upon antigen activation (Fig. 5*E*, *Left*) compared with healthy individuals. Of note, the expression of CD48 was also slightly reduced in MS patients (Fig. 5*E*, *Right*). Overall, our data proposed that defective cytolytic activity of NK cells in MS is most likely attributable to an impaired DNAM-1/CD155 and 2B4/CD48 interaction between NK cells and CD4$^+$ T cells.

Therapeutic Modulation of the IL-2R by DAC HYP Restores Impaired NK-Cell Immune-Regulatory Functions in MS Patients. So far, our data revealed that MS alters the NK-cell compartment on many different levels, including expression of certain activating

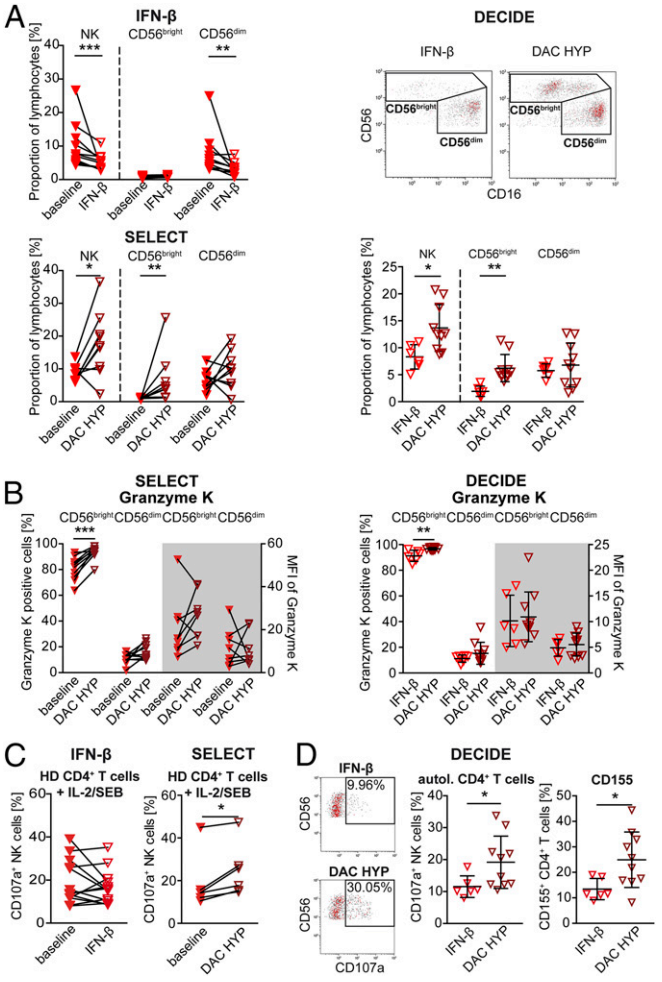

Fig. 6. Therapeutic IL-2R modulation in MS patients restores impaired immune-regulatory function of NK cells via up-regulation of the DNAM-1 ligand CD155. Phenotype and function of NK cells derived from MS patients before (closed red triangles down) and after 1-y treatment with IFN-β (open red triangles down) (n = 13) or with DAC HYP (open cayenne triangles down; SELECT study, n = 9) were investigated. Furthermore, phenotype and function of NK cells derived from 16 MS patients of the DECIDE study were investigated in a blinded fashion. Unblinding revealed that 6 patients had received IFN-β and 10 patients had received DAC HYP. (*A*) Proportions of NK cells and their subsets from the indicated trials are displayed. Representative CD16 versus CD56 plots of NK cells (*Upper Right*) from the DECIDE trial are shown. Wilcoxon matched-pairs signed rank test (IFN-β; SELECT) and unpaired Student's t test (DECIDE) were used. (*B*) Proportions and MFI of GrK-expressing CD56^{bright} and CD56^{dim} NK cells from the SELECT (*Left*) and DECIDE (*Right*) trials. Wilcoxon matched-pairs signed rank test (SELECT) and unpaired Student's t test (DECIDE) were used. (*C*) Degranulation of IL-15–stimulated (48 h) NK cells at baseline (closed red triangles down) and after 1-y treatment with IFN-β (open red triangles down) (n =13) or DAC HYP (open cayenne triangles down; SELECT study) (n = 6) in response to IL-2/SEB-activated allogeneic CD4$^+$ T cells derived from independent HDs. Paired Student's t test was used. (*D*, *Left* and *Center*) Representative CD107a versus CD56 plots of NK cells (*Left*) and proportions (*Center*) of degranulating IL-15–activated NK cells from the IFN-β (open red triangles) or DAC HYP (open cayenne triangles) arm of the DECIDE study in response to autologous (autol.) IL-2/SEB-activated (48 h) CD4$^+$ T cells (*Center*). (*D*, *Right*) Proportions of CD155$^+$ CD4$^+$ T cells derived from MS patients treated with either IFN-β (open red triangles down) or DAC-HYP (open cayenne triangles down) following 48 h of stimulation with IL-2/SEB. Unpaired Student's t test was used. *P < 0.05; **P < 0.01.

NK-cell receptors (Fig. 3) as well as cytolytic activity in response to CD4$^+$ T cells. To investigate the therapeutic effects of IL-2R modulation on the observed alterations, phenotype and function of NK cells derived from MS patients treated with DAC HYP

(the SELECT and DECIDE studies) were compared with those receiving IFN-β (IFN-β treated patients and patients from the DECIDE study). One-year treatment with IFN-β had no impact on the CD56^{bright} NK-cell subset (Fig. 6*A*, *Upper Left*), whereas frequencies of CD56^{bright} NK cells were significantly increased in MS patients treated with DAC HYP compared with baseline (Fig. 6*A*, *Lower Left*, SELECT study) and patients treated with IFN-β (Fig. 6*A*, *Right*, DECIDE study). Moreover, the proportions of GrK-expressing CD56^{bright} NK cells were slightly increased in response to DAC HYP (Fig. 6*B*, SELECT and DECIDE studies). Whereas treatment of MS patients with IFN-β had no impact on the degranulation of NK cells (Fig. 6*C*, *Left*), IL-2R modulation improved cytolytic activity of NK cells in response to allogeneic T cells (Fig. 6*C*, *Right*, SELECT study). Of note, the expression of the activating NK-cell receptors DNAM-1 and 2B4, which were expressed to a lower degree on the cell surface of MS patients (Fig. 3*B*), remained unaltered (Fig. S4*A*, SELECT study). So far, our setup only focused on the impact of IL-2R modulation on NK cells, because antigen-activated but otherwise "untreated" CD4$^+$ T cells were used as target cells. For further investigation of the therapeutic effects of IL-2R modulation on CD4$^+$ target cells, cytolytic activity of NK cells of MS patients treated with DAC HYP in response to autologous CD4$^+$ T cells was compared with that of MS patients treated with IFN-β (Fig. 6*D*, DECIDE study). NK cells of DAC HYP-treated patients revealed a significantly enhanced cytolytic activity in response to syngeneic antigen-activated CD4$^+$ T cells than those treated with IFN-β (Fig. 6*D*, *Left*), and accordingly frequencies of CD155 expressing CD4$^+$ T cells were also significantly increased under DAC HYP treatment (Fig. 6*D*, *Right*). Taken together, these data suggest that therapeutic immune modulation of the IL-2R at least partially restores impaired immune-regulatory function of NK cells in MS by both targeting the NK-cell as well as the T-cell target side (Fig. 7).

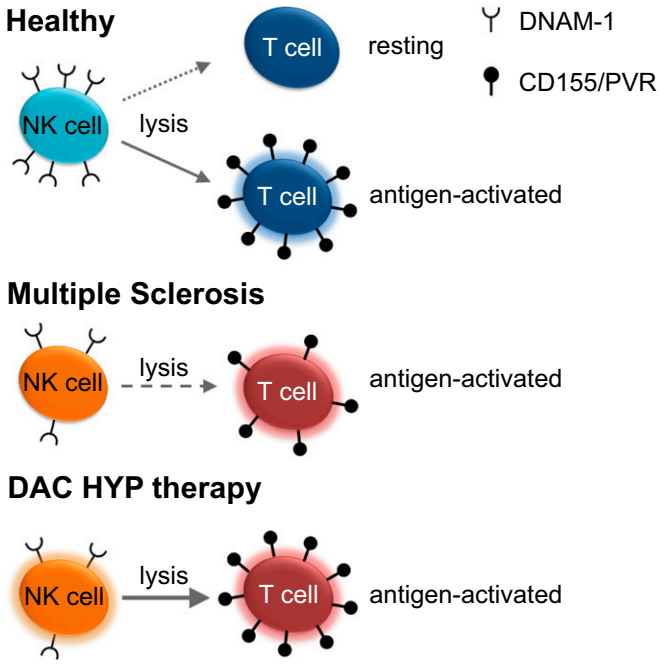

Fig. 7. Working hypothesis. Antigen-triggered up-regulation of the DNAM-1 ligand CD155 renders CD4$^+$ T cells more sensitive to NK cell-mediated lysis (*Left*). Impaired immune-regulatory functions of NK cells in MS patients are attributable to reduced up-regulation of CD155 on antigen-activated T cells on the one hand and lower expression of its receptor DNAM-1 on NK cells on the other hand (*Center*). Therapeutic immune modulation with DAC HYP restores defective immune-regulatory function of NK cells by both improving their cytolytic capacity as well as increasing CD155 expression on CD4$^+$ T cells (*Right*).

Discussion

The aim of the current study was to characterize the role of NK cells in human CNS autoimmunity and the impact of IL-2R modulation on NK-cell immune-regulatory functions. Investigating the presence, distribution, and function of NK cells in three different compartments (CNS, CSF, and PB), our data revealed an important role of NK cells in controlling T-cell activity in vivo and a deficit of NK-mediated control of T-cell activity in MS that could be restored by therapeutic modulation of the IL-2R. Thus, in addition to regulatory T cells and tolerogenic DCs, NK cells seem to be important players in controlling T-cell activation and MS-related impairment of this cell population might be one of the driving forces in the pathogenesis of this disease.

MS-related higher frequencies of the NK-cell activation marker NKp44-expressing CD56^{bright} NK cells suggest MS-related activity of this subset in vivo. Interestingly, NK cells seem not only control T-cell activity in the periphery but also in the CNS, because occurrence of immune-regulatory GrK-expressing NK cells in active MS lesions in close proximity to T cells indicates an active role of these cells in regulating T cell-mediated inflammation in the CNS. CSF analysis revealed that in accordance with the literature (42), the majority of intrathecal NK cells were CD56^{bright} NK cells, and both enhanced adhesion to and transmigration of this subset across primary HBMECs suggest that increased proportions of CD56^{bright} NK cells are at least partly attributable to their higher trafficking capacity across the BBB. Enhanced trafficking capacity could be an intrinsic feature of the CD56^{bright} NK-cell subset and accordingly static imaging of distinct NK-cell subsets added to HBMEC monolayers revealed a twofold-higher motility of CD56^{bright} NK cells compared with their CD56^{dim} counterparts. Moreover, HLA-C expressed on HBMECs (Fig. S5) might inhibit adhesion and trafficking of KIR-expressing CD56^{dim} NK cells through interaction with this inhibitory receptor as reported elsewhere (43), whereas CD56^{bright} NK cells are not inhibited, because HBMECs do not express the inhibitory NKG2-A ligand HLA-E (Fig. S5). Finally, reduction of intrathecal CD56^{bright} NK-cell frequencies to levels of healthy individuals in MS patients treated with the $\alpha_4\beta_1$ integrin blocker (44) natalizumab indicates that transmigration of NK cells into the CNS depends on VLA-4. The alternative MCAM-dependent pathway that has been recently described for Th17 (38) cells can be ruled out, because NK cells do not express this receptor. Of note, the trafficking capacity of CD56^{bright} NK cells was not impaired in MS patients and similar proportions of CD56^{bright} NK cells observed in the CSF of both MS patients as well as healthy individuals propose a general role of this NK-cell subset in CNS immune surveillance. Intrathecal CD56^{bright} NK-cell/CD4 T-cell ratios did not differ between MS patients and healthy individuals. Because the majority of intrathecal NK cells are immune-regulatory CD56^{bright} NK cells, a disease-accelerating role of NK cells in CNS autoimmunity as it has been proposed (45–48) is unlikely.

MS is associated with alterations of the peripheral NK-cell compartment. We found reduced cell surface expression of the activating NK-cell receptor DNAM-1 (CD226), a genetic alteration consistently found in MS-association studies (16–18), and 2B4. DNAM-1, the receptor for Nectin-2 (CD112) and PVR (CD155), is an activating NK-cell receptor, which is crucial for controlling NK-cell cytotoxicity (reviewed in ref. 49) and requires the coactivating receptor 2B4 for full NK-cell activation (40, 41, 50). Indeed, our data revealed that, at least for the T-cell stimulation protocol used in the current study, DNAM-1/CD155 as well 2B4/CD48 interactions seemed to be a prerequisite for NK-cell mediated control of CD4$^+$ T-cell activation (Fig. 7, *Top*). Although IL-2–activated CD4$^+$ T cells only expressed the 2B4 ligand CD48 on their cell surface and failed to activate NK-cell cytolytic function, stimulation of T cells with IL-2 and SEB resulted in an up-regulation of the DNAM-1 ligand CD155 concomitantly with an enhanced NK-cell cytolytic activity (Fig. 7, *Top*). Moreover, cytolytic activity of NK cells positively correlated with expression of CD155 on CD4$^+$ T cells and reduced up-regulation of CD155 expression on CD4$^+$ T cells using siRNA resulted in lower NK-cell response.

With regard to the siRNA experiments, one has to keep in mind the recently reported role of CD155 as a costimulatory molecule for $CD4^+$ T cells (51). Thus, CD155 knock down could also induce a suboptimal activation of $CD4^+$ T cells, resulting in a reduced susceptibility to NK-cell killing by other mechanisms than CD155. Strikingly, we observed a significantly reduced cell surface expression of both DNAM-1 as well as its coreceptor 2B4 on NK cells derived from MS patients, proposing that this might result in a MS-related decrease of NK-cell-mediated control on T-cell activity in vivo; however, it might be possible that other NK-cell receptors might also be involved. Furthermore, a recent study revealing a crucial role of DNAM-1 in NK-cell "licensing" (52) suggests that NK cells of MS patients might be less "licensed."

Our hypothesis that impaired DNAM-1/CD155 interactions in MS disrupts NK-mediated control of T-cell activity (Fig. 7, *Middle*) is further supported by genome-wide association studies (GWAS) revealing that a single nucleotide polymorphism in the *CD226* gene, leading to one amino acid substitution in the DNAM-1 receptor (Gly307Ser/rs763361^T), is associated with a higher incidence in developing several autoimmune diseases including MS (16–18). Although additional work is required to elucidate whether this amino acid substitution, which occurs in the intracellular domain of DNAM-1, affects DNAM-1 signaling and NK-cell cytolytic function (49), these data suggest that DNAM-1 plays critical role in controlling autoimmunity. MS-related reduced cell surface expression of DNAM-1 could be also attributable to down-modulation in response to sustained receptor engagement, because receptor down-modulation has been shown for impaired NK cell function in tumor control [e.g., down-regulation of DNAM-1 by CD155 expressing tumor cells (53)]. Binding of the CD226 antagonists CD96 and TIGIT (T-cell immunoreceptor with Ig and ITAM domains) result in decreased cytokine secretion (54) and degranulation (55) by NK cells, respectively. Thus, investigating the role of these two CD226 antagonists that are both expressed on human NK cells in the observed defective NK-mediated regulation of T-cell activity in MS is further warranted.

In addition to a higher threshold for NK-cell activation attributable to lower expression of DNAM-1 as well as a gene polymorphism in the *CD226* gene, our study clearly demonstrated that impaired immune regulation in MS was also attributable to a $CD4^+$ T-cell evasion mediated by lower up-regulation of the DNAM-1 ligand CD155 upon antigen activation (Fig. 7, *Middle*). Down modulation of CD155 seems to be a common concept used by $CD4^+$ T cells to escape NK cell-mediated control. In HIV-1–infected $CD4^+$ T cells, the early viral Nef protein down-modulates CD155 expression, thereby preventing NK cell-mediated lysis of infected T cells (15). Of note, NK-cell cytolytic activity toward antigen-activated MHC class I-expressing $CD4^+$ T cells seems to be impaired in MS, whereas no deficit in response to MHC class I-deficient tumor target cells was observed. MHC class I deficiency lowers the threshold for NK-cell activation tremendously (13), and therefore lower expression of certain activating NK-cell receptors in MS might be negligible.

MS seems not only to alter NK-cell immune-regulatory functions but also their differentiation. It is well accepted that NK cells differentiate from the more immature $CD56^{bright}$ to the mature $CD56^{dim}$ stage (56, 57). We found a slight but significant decrease of peripheral NK cells in MS, a finding that was also observed in earlier studies (56, 57). We could further show that the decrease is attributable to a reduction of the $CD56^{dim}$ NK-cell subset, whereas frequencies of $CD56^{bright}$ NK cells remained unaltered, thus suggesting that NK-cell maturation might be impaired in MS. The finding that therapeutic immune modulation of the IL-2R with daclizumab significantly enhances frequencies of the immature $CD56^{bright}$, but not the mature $CD56^{dim}$ NK-cell subset (10), further supports this hypothesis. It is likely that although daclizumab promotes differentiation of $CD56^{bright}$ NK cells from a common innate lymphoid cell (ILC) progenitor (58), NK cells then get trapped in the more immature $CD56^{bright}$ stage because of a failure in further maturation. Observed lower frequencies in IL-2Rα as well as β-chain–expressing $CD56^{bright}$ NK cells in MS patients might be a possible explanation for diminished NK-cell maturation, because among others, the IL-2R drives NK-cell maturation. Furthermore, cytokines secreted by activated T cells could also suppress the $CD56^{dim}$ NK-cell subset. It has been shown that IL-21 secreted by antigen-activated T cells including Th17 cells enhances NK-cell function (59, 60) but antagonizes NK-cell viability, thus indicating a role for this cytokine in promoting NK-cell degeneration by autoreactive T cells (61).

DAC HYP is a new promising MS therapy under investigation, which recently demonstrated superior efficacy relative to IFN-β on annualized relapse rate as well as MRI lesions in a phase III clinical study [the DECIDE study (32)]. It has been postulated that DAC HYP largely acts via modulation of NK-cell function (reviewed in ref. 4). In our study, we had the unique opportunity to analyze immune cells from MS patients, who participated in the DECIDE trial and received either DAC HYP or IFN-β as part of the study protocol. Moreover, we analyzed biomaterial from MS patients before and following 12 mo of DAC HYP treatment as part of the SELECT study (62). Our data revealed that therapeutic modulation of the IL-2R with DAC HYP induced changes in NK-cell phenotype and function. Frequencies of $CD56^{bright}$ NK cells were significantly increased in DAC HYP-treated MS patients in comparison with both baseline levels (the SELECT study) as well as patients of the IFN-β arm (the DECIDE study). Interestingly, frequencies of NKp44-expressing (a surrogate marker for NK-cell activation) $CD56^{bright}$ NK cells were also enhanced following 1-y treatment with DAC HYP (the SELECT study), indicating that immune modulation of the IL-2R triggers activity of this NK-cell subset in vivo.

IFN-β is a well-established first-line therapy for treatment of MS that has also been suggested to have an impact on the NK-cell compartment, but to date IFN-β's effect on NK-cell immune-regulatory function is still poorly understood (22–24, 63). Comparing patients before and following 1-y treatment with DAC HYP (the SELECT study) with those treated with IFN-β demonstrated that DAC HYP increased NK-cell cytotoxicity, whereas it remained unaltered in MS patients treated with IFN-β, suggesting that the latter treatment has no impact on NK-cell immune-regulatory function. Along these lines, we could also show that NK-cell cytolytic activity in response to antigen-activated $CD4^+$ T cells was significantly enhanced in MS patients treated with DAC HYP relative to IFN-β (the DECIDE study). In correlation with the recently demonstrated superior efficacy of DAC HYP compared with IFN-β [the DECIDE study (32)], our finding further supports the hypothesis that NK cells are crucial players in controlling T-cell activity. Moreover, NK cells might even reduce the dependency on T-cell immune-regulatory functions, because regulatory T cells are strongly decreased in patients treated with DAC HYP, despite its therapeutic benefit (10, 62).

DAC HYP restores impaired immune-regulation by targeting both the NK-cell as well as the T-cell side (Fig. 7, *Bottom*). On the one hand, IL-2R modulation resulted in an increased cytolytic capacity of NK cells [in accordance with the literature (10, 30, 31, 33)]. The puzzling finding that neutralization of the IL-2Rα chain results in enhanced cytotoxicity of NK cells might be attributable to a higher availability of T cell-derived IL-2 for NK-cell activation, as has been proposed by others (30). Nevertheless, our study suggests that another mechanism is also likely. We could demonstrate that DAC HYP-mediated increased activation of NK cells depends on the presence of DCs. DCs have been shown to cross-present IL-2 and IL-15 to lymphocytes via the IL-2Rα (64) and IL-15Rα chain (57, 65, 66), respectively. Both cytokines are transpresented to and signal through the IL-2Rβ and common γ-chain, and blocking IL-2Rα cross-presentation using DAC HYP might therefore result in enhanced IL-15 signaling in NK cells, leading to the increased cytolytic activity. On the other hand, we observed significantly higher frequencies of CD155-expressing $CD4^+$ T cells upon antigen activation in MS patients treated with DAC HYP compared with those of the IFN-β arm (the DECIDE study). Therefore, immune modulation of the IL-2R does not only trigger NK-cell cytotoxicity but also seems to restore impaired DNAM-1/CD155

interaction in MS patients by rendering $CD4^+$ T cells more sensitive to NK cell-mediated lysis (Fig. 7, *Bottom*). This hypothesis is further supported by an earlier study suggesting that activated T cells derived from MS patients under daclizumab therapy seem to be more sensitive to NK-mediated lysis than activated T cells from baseline (10).

In conclusion, our data postulate that functionally disturbed immune-regulatory networks of NK cell-mediated T-cell regulation in CNS autoimmunity is caused by impaired DNAM-1/CD155 interaction and restored by therapeutic modification of the IL-2R with DAC HYP.

Materials and Methods

Detailed methods are provided in *SI Materials and Methods*.

Patients and Healthy Controls. A total of 182 patients with the diagnosis of clinically isolated syndrome (CIS) or clinically definite relapsing-remitting MS (RRMS) according to the 2010 revised McDonald diagnostic criteria (67) were enrolled in this study. Patient demographics are summarized in Table S1. Human tissue samples from seven MS patients (paraffin-embedded section of three MS patients; frozen sections of four MS patients) were investigated retrospectively. None of the study authors was involved in decision-making with respect to biopsy. The study was performed in accordance with the declaration of Helsinki and approved by the local ethics committees of the Universities of Dresden, Münster, and Munich (LMU Munich, registration no. 245/02; Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Medizinischen Fakultät der Westfälischen Wilhelms-Universität Münster; registration nos. 2010-262-f-S and 2012-236-f-S). For patient samples derived from the SELECT trial, institutional review board/ethics committee approval was obtained at each participating center (62). Lumbar puncture was performed for medical reasons only. All participants of this study provided written informed consent.

Immunohistochemistry. All lesions fulfilled the generally accepted criteria for the diagnosis of MS (67). Demyelinating activity was classified as described in detail earlier (68).

Tissue specimens were fixed in PBS supplemented with 4% (wt/vol) paraformaldehyde and embedded in paraffin. Tissue samples were cut in 4-μm-thick sections that were stained with hematoxylin/eosin (H&E) and Luxol-fast blue. Immunohistochemical staining was performed with an avidin–biotin technique using an automated staining device (DakoLink 48). For the detection of NK and T cells in the lesions of MS patients, sections were treated with EnVision FLEX (DAKO) target retrieval solution; probed with anti-CD56 (123C3; DAKO), anti-GrK (69884; Abcam), and anti-CD3 (EPR4517; Epitomics); and analyzed with Axio Scope.A1 (Zeiss).

Cells and Cell Culture. Blood was withdrawn into EDTA-containing tubes (K2E Vacutainer; BD), and PBMCs were isolated by density centrifugation and cryopreserved in liquid nitrogen according to our standard operating procedures (SOPs) (69). Frozen PBMCs from the SELECT and DECIDE study were collected according to the respective study protocols and obtained from study investigators by shipping in sealed liquid nitrogen containers with continuous temperature surveillance. Frozen PBMCs were thawed according to our SOPs as previously described (69). NK cells were isolated from freshly isolated PBMCs using the NK-cell enrichment kit (Stem Cell Technologies) (70).

SEB-activated (Sigma) $CD4^+$ T cells (SEB a$CD4^+$ T cells) were generated by stimulation of thawed PBMCs in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% (vol/vol) heat-inactivated FCS, 200 IU/mL recombinant human IL-2 (Peprotech), and 100 μg/mL SEB at cell densities of 2×10^6 cells/mL. Following 4 d of stimulation at 37 °C and 5% (vol/vol) CO_2 $CD4^+$ T cells were isolated using the CD4 T Cell Isolation Kit II (Miltenyi) according to the manufacturer's instructions.

Flow Cytometry. For labeling of cell surface molecules, PBMCs were stained with fluorochrome-conjugated antibodies diluted in PBS/0.5% BSA/2 mM EDTA and incubated for 45 min at 4 °C at the indicated working concentrations (Table S2). For intracellular staining of perforin and GrK, cell surface-labeled PBMCs were permeabilized with fixation/permeabilization solution (eBiosciences), followed by intracellular staining with fluorochrome-conjugated anti-GrK and anti-perforin diluted in permeabilization solution (eBiosciences) at the indicated working concentrations (Table S2) according to the manufacturer's instructions. NKp30, NKp44, and NKp46 ligands were stained with 10 μg/mL of the respective Fc-fusion protein (R&D Systems) as described above. Following extensive washing, bound fusion proteins were labeled with 1:50 diluted FITC-conjugated goat anti-human IgG. Flow cytometry of CSF and whole blood cells was performed as previously described (71). All flow cytometry data were acquired on a Gallios (Beckman Coulter) or Navios (Beckman Coulter) flow cytometer, and the resulting data were analyzed using Kaluza Flow Cytometry Analysis version 1.2 software (Beckman Coulter) and Prism version 5.04 software (GraphPad).

Static Imaging of NK Cells. Static imaging of $CD56^{bright}$ and $CD56^{dim}$ NK-cell subsets was performed as previously described (38).

Conjugation Assay. Conjugation assays were performed as previously described (72).

Migration Assay. Migration assays were performed as previously described (Fig. S2 and ref. 73). The confluence of the HBMEC monolayer was monitored by testing its permeability for Evans Blue (Sigma) (Fig. 2*B*). HBMECs were stimulated with 500 IU/mL IFN-γ (Peprotech) and TNF-α (Peprotech) 24 h prior to the assay. PBMCs were added to the upper compartment, and the transmigration of NK-cell subsets across the HBMEC monolayer after 6 h was determined by flow cytometry. A well containing 5×10^5 thawed PBMCs in 700 μL of RPMI-1640/2% B27 served as an in vitro control. The percentage of migrated cells for each cell type was calculated after the following equation:

$$\text{migrated cells}[\%] = (\text{absolute number of cells lower compartment} \times 100)/\text{absolute number of cells in vitro}.$$

Degranulation Assay. Release of cytolytic granules by NK cells was determined using an effector to target cell ratio of 1:1 as previously described (70).

Silencing of CD155 Expression in SEB-Activated Primary Human $CD4^+$ T Cells. SEB-activated $CD4^+$ T cells were generated as described above. Cells were harvested on days 2 and 3 and transfected with either CD155-specific siRNA [5′-CAACUUUAAUCUGCAACGUdTdTUU-3′ (74)] or ON-TARGETplus non-targeting siRNA #1 (both from ThermoScientific) by electroporation using the BTX ECM 830 Square Electroporator.

Statistics. Data were analyzed using the Prism version 5.04 software (GraphPad). The D'Agostino–Pearson omnibus normality test was performed to test for Gaussian distribution. Depending on the result, unpaired Student's *t* test or Mann–Whitney test was used to compare means between two independent groups, whereas paired Student's *t* test or Wilcoxon matched-pairs signed rank test was used for different treatments within the same patient group and for comparisons between PB and CSF. >Two datasets were compared using Kruskal–Wallis with Dunn's posttest. Correlation was analyzed by Pearson test. Data depicted in the figures display means ± SD. Box plots show the median with whiskers depicting minimal to maximal values. Differences were considered statistically significant with the following *P* values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

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