



Acetylcholine-producing NK cells attenuate CNS inflammation via modulation of infiltrating monocytes/macrophages

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The nonneural cholinergic system of immune cells is pivotal for the maintenance of immunological homeostasis. Here we demonstrate the expression of choline acetyltransferase (ChAT) and cholinergic enzymes in murine natural killer (NK) cells. The capacity for acetylcholine synthesis by NK cells increased markedly under inflammatory conditions such as experimental autoimmune encephalomyelitis (EAE), in which ChAT expression escalated along with the maturation of NK cells. ChAT⁺ and ChAT⁻ NK cells displayed distinctive features in terms of cytotoxicity and chemokine/cytokine production. Transfer of ChAT⁺ NK cells into the cerebral ventricles of CX3CR1^{-/-} mice reduced brain and spinal cord damage after EAE induction, and decreased the numbers of CNS-infiltrating CCR2⁺Ly6C^{hi} monocytes. ChAT⁺ NK cells killed CCR2⁺Ly6C^{hi} monocytes directly via the disruption of tolerance and inhibited the production of proinflammatory cytokines. Interestingly, ChAT⁺ NK cells and CCR2⁺Ly6C^{hi} monocytes formed immune synapses; moreover, the impact of ChAT⁺ NK cells was mediated by α 7-nicotinic acetylcholine receptors. Finally, the NK cell cholinergic system up-regulated in response to autoimmune activation in multiple sclerosis, perhaps reflecting the severity of disease. Therefore, this study extends our understanding of the nonneural cholinergic system and the protective immune effect of acetylcholine-producing NK cells in autoimmune diseases.

acetylcholine | NK cell | CCR2⁺Ly6C^{hi} monocyte | immune homeostasis | EAE

Inflammation and immune responses within the central nervous system (CNS) are the causative features of multiple sclerosis (MS) (1), which afflicts ~2.5 million individuals worldwide. MS and its animal model, experimental autoimmune encephalomyelitis (EAE), are characterized by immune cell infiltration of the CNS, demyelination, and axonal damage, thus leading to functional paralysis (2).

The cholinergic antiinflammatory system plays a well-established role in maintaining immune homeostasis in various inflammatory or autoimmune diseases (3, 4). The expression of choline acetyltransferase (ChAT) and synthesis of acetylcholine (ACh) have been proven in multiple immune cells, such as CD4⁺ T cells, B cells, dendritic cells, and macrophages, by either inflammatory or neuronal stimulation; in turn, the presence of ChAT and ACh can outweigh the overactivated immune response (5, 6). During maturation, some immune cells gain the ability to synthesize ACh, which is an important participant in controlling the immune response through interaction with regional innate immune cells (6). However, whether NK cells express ChAT within organ-specific inflammatory environments during development and, if so, its role in immunity remain unclear.

The impact of NK cells on the outcome of CNS autoimmunity varies depending on the course of disease progression (7–10). The CD56^{bright} subset of human NK cells was shown to provide immune protection under MS conditions, the effect of which was proven and amplified by daclizumab or daclizumab high-yield process treatment (11–16). CNS-resident NK cells mediate immune suppression during the acute stage of CNS autoimmune

diseases via cross-talk with microglia/macrophages of the CNS (17, 18). The reciprocal interaction between NK cells and monocytes/macrophages of the innate immune system has also been considered crucial for the immune activity of these cells in various inflammatory conditions (19–22). However, a more profound understanding of the detrimental and beneficial roles of macrophages in EAE has been reported, which relies on the new recognition of monocyte-derived macrophages being functionally distinct from microglia-derived macrophages (23–27). Among these cells, CCR2⁺Ly6C^{hi} monocytes, which are rapidly recruited to the CNS and further transform into macrophages, proved to be crucial in EAE progression (24, 28, 29). The Ly6C^{hi} subset of monocytes was described as the most potent for NK cell activation (30). This background led us to reinvestigate the interaction between NK cells and the damage-evoking macrophages in EAE. Our previous work on EAE documented the immune protective effect of cholinergic antiinflammatory action, shown by inhibiting immune cell activation and inflammatory cytokine secretion (31–33). Additionally, we recently found that the migration of CCR2⁺Ly6C^{hi} monocytes was largely inhibited by nicotine treatment, resulting from the effect of exogenous cholinergic antiinflammatory activity (34, 35). However, the existence of an

Significance

Acetylcholine (ACh) produced by neurons performs an array of functions that control cardiac, gastrointestinal, and other biosystems. Here we discovered that lymphocytic natural killer (NK) cells bear machinery that produces ACh. The activity of ACh-producing NK cells up-regulates during the disease flare of multiple sclerosis (MS) and may, therefore, reflect the pathologic state. In the mouse model of MS, experimental autoimmune encephalomyelitis, these ACh-producing NK cells can reduce the intensity of inflammation and autoimmune responses in the brain and spinal cord. Therefore, the nonneural cholinergic system, as reflected by ACh-producing NK cells, appeared to counteract aberrant immune responses and lessen brain damage. This observation offers insight into the therapeutic mechanisms of the Food and Drug Administration-approved drug daclizumab high-yield process for MS.

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endogenous cholinergic system in NK cells and its possible role in mediating the immune effect on infiltrating monocyte-derived macrophages are uncertain. Therefore, this study was performed to determine whether the cholinergic system exists in NK cells and, if so, how it contributes to an immune protective effect during inflammation.

Results

The Cholinergic System in NK Cells and Its Activation upon Immune Stimulation. NK cells from the spleens of mice were obtained by FACS sorting based on NK1.1 and CD3 expression. The purity of NK cells (NK1.1⁺CD3⁻) was above 95% after sorting (Fig. 1A). Total mRNA was extracted from the sorted NK cells, followed by reverse transcription. The expression of ChAT, vesicular ACh transporter (VACHT), acetylcholinesterase (AChE), and choline transporter 1 (CHT1) in murine NK cells was confirmed by amplified cDNA electrophoresis, with samples from brain cells used as a positive control (Fig. 1B). Western blot analyses were further conducted to identify the protein expression of ChAT, VACHT, AChE, and CHT1 in NK cells (Fig. 1C). To determine the capacity for ACh synthesis in NK cells, an established method for measuring concentrations was applied, with *d*₉-ACh used as the internal standard (36) (Fig. 1D). The detection limit of ACh concentration ranged from 0.05 to 5 ng/mL (Fig. 1E). When intracellular ACh production was quantified in NK cells by ultraperformance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS), IL-2 and LPS stimulation of the cultured NK cells proved to increase their ACh production (0.06 ± 0.03 vs. 0.22 ± 0.07 ng/10⁶ cells for control vs. IL-2 + LPS; $P < 0.05$) (Fig. 1F). Immune cells were isolated from the spleens of wild-type (WT) and ChAT-enhanced green fluorescent protein (eGFP) mice, and ChAT expression in total splenic immune cells as well as NK cells was measured based on eGFP level. Once again, we identified the presence of ChAT⁺ immune cells in spleens from ChAT-eGFP mice compared with spleen cells from WT mice (6) (Fig. 1G). Interestingly, we found that a small portion of NK cells was ChAT⁺ and that the expression increased upon IL-2 and LPS stimulation in vitro. IL-2 alone had no obvious effect on ChAT expression in NK cells; however, LPS activation increased ChAT expression significantly in culture ($0.98 \pm 0.17\%$, $1.18 \pm 0.24\%$, and $2.98 \pm 0.45\%$ for control, IL-2 treatment, and IL-2 + LPS treatment, respectively; $P < 0.05$ when comparing control vs. IL-2 + LPS treatment or IL-2 vs. IL-2 + LPS treatment) (Fig. 1H). This ChAT-eGFP was expressed in NK cells constitutively, and LPS stimulation increased the expression markedly within 12 h of culture ($0.31 \pm 0.10\%$ vs. $0.98 \pm 0.17\%$, $0.22 \pm 0.07\%$ vs. $2.48 \pm 0.30\%$, $0.15 \pm 0.04\%$ vs. $2.98 \pm 0.45\%$, and $0.47 \pm 0.22\%$ vs. $2.73 \pm 0.32\%$, respectively, for WT vs. ChAT-eGFP⁺ mice at 0, 12, 24, and 36 h; $P < 0.05$ for each time point) (Fig. 1I). These data confirm the existence of a cholinergic system in NK cells and its ability to up-regulate ACh synthesis during the acute phase of inflammatory stimulation.

Induction of ChAT-eGFP During NK Cell Development and Phenotypic Maturation upon EAE Induction. To investigate the development of ChAT expression in NK cells and the underlying mechanisms of the cholinergic system's activation in response to inflammatory stimulation, we induced EAE in ChAT-eGFP mice and assessed ChAT-eGFP expression in splenic NK cell subsets. The development of NK cells can be divided into four stages based on CD11b expression in combination with CD27: CD11b⁻CD27⁻, CD11b⁻CD27⁺, CD11b⁺CD27⁺, and CD11b⁺CD27⁻ (Fig. 2A) (37, 38). ChAT-eGFP expression was evaluated in each of these NK cell subsets from the spleen in normal and EAE conditions. ChAT-eGFP expression increased during NK cell maturation in normal conditions. The percentage of ChAT⁺ NK cells in the CD11b⁻CD27⁻ subset was the lowest, whereas ChAT-eGFP expression in CD11b⁺CD27⁻ was higher than in any other subset. EAE induction further enhanced

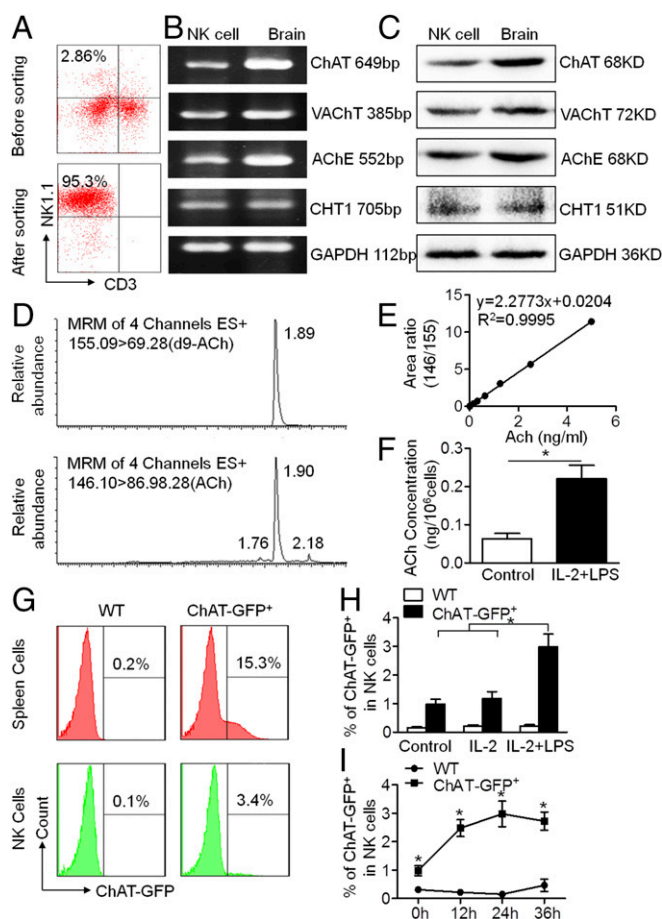


Fig. 1. Cholinergic system exists in murine NK cells, and acetylcholine synthesis increases under inflammatory stimulation. (A) NK cells (NK1.1⁺CD3⁻) from the spleens of C56BL/6 mice were sorted by FACS, producing a purity of above 95%. (B and C) Total mRNA and protein of sorted NK cells were extracted to identify key components of the cholinergic system by RT-PCR and Western blot, compared with the positive control from brain samples. Choline acetyltransferase, vesicular acetylcholine transporter, acetylcholinesterase, and choline transporter 1 were constitutively expressed and synthesized by murine NK cells. $n = 5$. (D–F) The concentration of intracellular ACh was determined by UPLC-MS/MS with *d*₉-ACh as the internal standard. Intracellular ACh production by NK cells increased under LPS stimulation with IL-2. $n = 6$ per group from two independent experiments. MRM, multiple reaction monitoring. (G–I) ChAT-eGFP expression in immune cells was counted by FACS. Percentages of ChAT⁺ spleen immune cells gated by forward and side scatter or spleen NK cells (NK1.1⁺CD3⁻) were detected after culture. LPS enhanced ChAT⁺ expression in NK cells above that in NK cells cultured with IL-2 alone or vehicle control. The percentage of ChAT⁺ NK cells increased when cultured with IL-2 and LPS and peaked at 24 h following treatment. $n = 4$ to 6 per group from three independent experiments. Mean \pm SEM. * $P < 0.05$.

ChAT-eGFP expression in the CD11b⁺CD27⁺ and CD11b⁺CD27⁻ subsets (Fig. 2B and C).

The distribution of ChAT⁺ NK cells changed dynamically with EAE progression. The progression of EAE over time is shown in Fig. 2D. NK cells originate in the bone marrow and then migrate into the peripheral blood and spleen, before finally infiltrating the CNS (8). The percentage of ChAT⁺ NK cells in bone marrow cells (BMCs) did not change obviously during the course of EAE. However, the ChAT-eGFP expression of NK cells in the periphery reached a peak at 7 d post immunization (dpi), followed by a slight decrease at 12 dpi, and then fell back to normal levels at 16 dpi (Fig. 2E). Meanwhile, the percentage of ChAT⁺ NK cells in the CNS reached the highest point at 16 dpi and fell at 20 dpi (Fig. 2F).

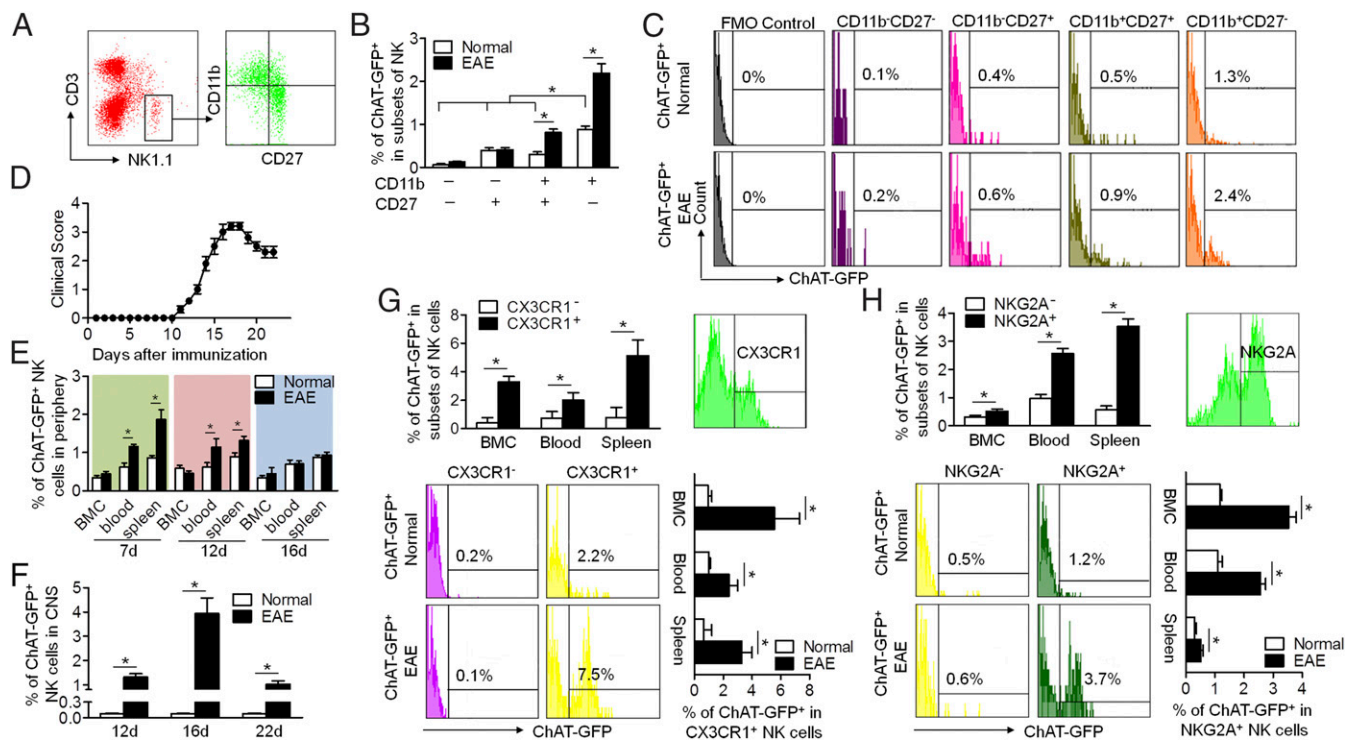


Fig. 2. ChAT-eGFP expression in murine NK cells during their development and phenotypic maturation upon EAE induction. (A) The development of NK cells is divisible into four stages based on the CD11b expression in combination with CD27. (B and C) ChAT-eGFP expression was determined on subsets of splenic NK cells at different development stages (CD11b⁻CD27⁻, CD11b⁻CD27⁺, CD11b⁺CD27⁻, CD11b⁺CD27⁺) in EAE mice. FMO control, fluorescence minus one control. ChAT expression increased during the maturation of NK cells under both normal and EAE conditions. In the presence of EAE, ChAT expression by mature NK cells increased dramatically. $n = 6$ per group. (D) The disease course of EAE was recorded: Initial manifestations occurred at 11 dpi; the peak was at around 16 dpi; and partial recovery was at 20 to 22 dpi. $n = 6$ per group. (E) During the course of EAE, the percentage of ChAT⁺ NK cells in the periphery (blood and spleen) changed dynamically with disease progression, peaked at 7 dpi, recovered slightly at 12 dpi, and returned to normal at 16 dpi. During that time, ChAT-eGFP expression remained constant in naive mice. $n = 6$ per group. (F) The percentage of ChAT⁺ NK cells that infiltrated into the CNS reached a peak at 16 dpi and fell at 20 dpi in EAE mice. $n = 12$ per group. (G) Circulatory NK cells were divided phenotypically into subsets based on CX3CR1 expression. The expression of ChAT-eGFP in CX3CR1⁺ NK cells exceeded that of CX3CR1⁻ NK cells. The EAE status increased ChAT-eGFP expression in CX3CR1⁺ NK cells. $n = 6$ per group. (H) Functional NK cells from the CNS were divided phenotypically into subsets based on NKG2A expression. The expression of ChAT-eGFP in NKG2A⁺ NK cells surpassed that of NKG2A⁻ NK cells. The EAE disease state increased ChAT-eGFP expression in NKG2A⁺ NK cells. $n = 6$ per group from three independent experiments. Mean \pm SEM. * $P < 0.05$.

This result indicated that ChAT expression in NK cells is closely related to their migration during EAE progression.

The dynamic distribution of ChAT⁺ NK cells in the periphery and CNS was associated with their migration during the disease course. NK cell homing to the CNS is largely dependent on the chemokine receptor CX3CR1 (18, 39, 40). Therefore, circulatory NK cells in the presence of EAE were phenotypically divided into two subsets based on CX3CR1 expression. Subsequent analysis revealed that ChAT-eGFP expression in CX3CR1⁺ NK cells was higher than that of CX3CR1⁻ NK cells from bone marrow, blood, and spleen. Additionally, the ChAT-eGFP expression in CX3CR1⁺ NK cells increased upon EAE induction (Fig. 2G). The phenotype of a CNS-infiltrated NK cell determines its role in the immune activity of EAE (11, 17). Functional NK cells from the CNS of EAE animals were divided based on NKG2A expression. The percentage of ChAT-eGFP⁺ expression in NKG2A⁺ NK cells was higher than that of NKG2A⁻ NK cells. The ChAT-eGFP expression in NKG2A⁺ NK cells then increased upon EAE induction (Fig. 2H). Overall, these data indicate that ChAT expression is induced during NK cell maturation and is associated with NK cell subtypes that possess specific mobility and function in the EAE environment.

ChAT Expression Is Associated with a Distinct Gene-Expression Profile of NK Cells. To gain more evidence of the potential functional difference between ChAT⁺ NK cells and ChAT⁻ NK cells, an mRNA microarray procedure was conducted. This method enabled analysis of these cells' gene-expression patterns. Interestingly, a clear de-

lineation between ChAT⁺ NK cells and ChAT⁻ NK cells was observed, with a total of 300 genes overexpressed and 941 genes underexpressed in ChAT⁺ NK cells (Fig. 3A). The difference in expression was defined as more than a twofold change ($P < 0.05$) (Fig. 3B), demonstrating that ChAT⁺ NK cells and ChAT⁻ NK cells have distinctly different gene profiles. To better understand the meaning of this outcome, we analyzed gene ontology (GO) in terms of clusters of up-regulated or down-regulated genes using the DAVID (Database for Annotation, Visualization and Integrated Discovery; <https://david.ncifcrf.gov/>) functional annotation tool. The differentially expressed genes belonged to several functional families, including those of NK cell cytotoxicity, chemokines, cytokines, and other components of immune responses. ChAT⁺ NK cells mainly overexpressed genes related to cytokines and chemokines, which are critically involved in mediating immune responses and chemotaxis under inflammatory conditions ($P < 0.05$). However, the down-regulated genes in ChAT⁺ NK cells were most relevant to the cytotoxicity of NK cells according to GO and Kyoto Encyclopedia of Genes and Genomes analysis ($P < 0.05$), which indicated the potential immune regulatory role of this subset (Fig. 3C). Overall, ChAT⁺ NK cells appeared to display a more activated phenotype toward immune regulation than the ChAT⁻ counterparts in preserving homeostasis.

ChAT⁺ NK Cells Reduce Pathologic Damage and Improve Neural Functions of EAE Mice. Because ChAT⁺ NK cells clearly differed from ChAT⁻ NK cells based on gene expression, further work was conducted to shed light on the functions of those cells in

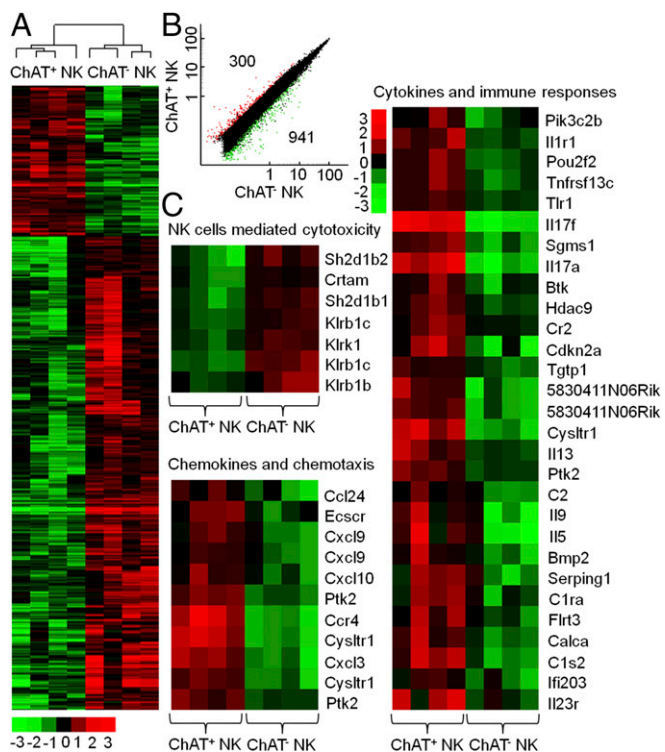


Fig. 3. mRNA microarray analysis reveals a distinctive expression profile of ChAT⁺ NK cells. ChAT⁺ NK cells and ChAT⁻ NK cells were sorted by flow cytometry based on eGFP expression to identify their genetic differences. (A) A total of 300 genes was overexpressed and 941 genes underexpressed in ChAT⁺ NK cells compared with ChAT⁻ NK cells, with a clear delineation. (B) The two groups differed in expression by more than twofold, with a corrected P value < 0.05 . (C) According to gene ontology analysis with the DAVID functional annotation tool, genes expressed in ChAT⁺ NK cells represented an up-regulation in immune responses and chemotaxis but a decrease in cytotoxicity. $n = 4$ per group.

EAE. In CX3CR1^{-/-} mice with EAE, NK cells failed to be recruited into the CNS, thus exacerbating disease severity (39). CX3CR1^{-/-} mice without CNS-infiltrating NK cells exhibited similar EAE severity to WT mice subjected to systemic NK cell depletion, indicating the crucial immune protective role of CNS-infiltrating NK cells rather than NK cells in the periphery (32). Thus, in our experiments, ChAT⁻ NK cells and ChAT⁺ NK cells (both from mature CD11b⁺CD27⁻ NK cell populations) were transplanted into the cerebroventricles of CX3CR1^{-/-} mice, which showed relieved disease severity, although ChAT⁺ NK cell implantation showed enhanced immune protective effects compared with ChAT⁻ NK cells (Fig. 4). CX3CR1^{-/-} mice showed earlier disease onset and higher scores for clinical symptoms of EAE from 12 dpi and lasting until 22 dpi. However, ChAT⁺ NK cell implantation delayed disease onset and reduced clinical scores in CX3CR1^{-/-} mice more significantly than ChAT⁻ NK cells (Fig. 4A–C). When 7-T MRI scans were conducted to detect demyelinated lesions in the brains and spinal cords of EAE mice, increased signal intensity was present in the periventricular region of the brain and lumbar enlargement of the spinal cord. The cumulative lesion volumes showed less demyelination in the ChAT⁺ NK cell-implanted groups ($3.05 \pm 0.32/\text{mm}^3$, $2.15 \pm 0.12/\text{mm}^3$, and $1.14 \pm 0.13/\text{mm}^3$ for vehicle, ChAT⁻ NK cell, and ChAT⁺ NK cell implantation, respectively; $P < 0.05$) (Fig. 4D and E). Hematoxylin & eosin (H&E) staining and Luxol fast blue (LFB) staining were then performed on spinal cord samples to visualize cell infiltration and demyelination. Cumulative results showed less accumulation of infiltrating cells ($47.14 \pm 2.86\%$, $36.86 \pm 1.96\%$, and $28.56 \pm 1.37\%$ for vehicle, ChAT⁻ NK cell, and ChAT⁺ NK cell implantation; $P < 0.05$) and demyelination after ChAT⁺ NK cell implantation ($28.86 \pm 2.20\%$,

$20.29 \pm 1.73\%$, and $11.43 \pm 1.51\%$ for vehicle, ChAT⁻ NK cell, and ChAT⁺ NK cell implantation; $P < 0.05$). After immunofluorescent staining with myelin basic protein (MBP), we witnessed aggravated demyelination along with a greater accumulation of cells, as indicated by DAPI staining in this lesion. However, ChAT⁺ NK cell implantation relieved this damage from demyelination ($37.29 \pm 3.40\%$, $27.63 \pm 3.29\%$, and $14.36 \pm 1.56\%$ for vehicle, ChAT⁻ NK cell, and ChAT⁺ NK cell implantation; $P < 0.05$) (Fig. 4F–I). Therefore, this outcome indicates that ACh-producing NK cells act as an important subset of NK cells in modulating immunity in CNS autoimmune disease.

ChAT⁺ NK Cells Diminish Accumulated CCR2⁺Ly6C^{hi} Monocytes and Modify the Cytokine Microenvironment in the CNS. CNS demyelination is initiated by immune cell infiltration and subsequent inflammatory responses of innate and adaptive immunity within the CNS (41). To understand the potential interactions of ChAT⁺ NK cells with CNS resident or infiltrative cells in mediating immune protection, we conducted further analysis. After ChAT⁺ NK cell implantation into the CNS of EAE mice, the content of infiltrating neutrophils (CD11b⁺Ly6G⁺), CD8⁺ T cells (CD3⁺CD8⁺), B cells (CD3⁻CD19⁺), and resident microglia (CD11b⁺CD45^{low}) did not change significantly (Fig. 5A and B); however, the total number of CD4⁺ T cells (CD3⁺CD4⁺) tended to decrease ($14.97 \pm 1.4\%$ vs. $10.72 \pm 1.73\%$; $P = 0.083$). Additionally, fewer total numbers of monocytes (CD11b⁺CD45^{hi}) were present within the CNS of mice that received ChAT⁺ NK cell implantation than ChAT⁻ NK cell implantation ($7.82 \pm 1.33\%$ vs. $12.07 \pm 1.15\%$; $P < 0.05$) (Fig. 5A and B). CCR2⁺Ly6C^{hi} monocytes (CD11b⁺Ly6G⁺CCR2⁺Ly6C^{hi}) are known to play an important role in the pathogenesis of EAE soon after infiltrating into the CNS (24, 28, 29). In our hands, flow cytometry showed large numbers of CCR2⁺Ly6C^{hi} monocytes in the CNS of the CX3CR1^{-/-} group but far fewer in the ChAT⁺ NK cell-implanted groups ($20.00 \pm 3.38\%$, $11.45 \pm 0.84\%$, and $5.97 \pm 1.33\%$ for vehicle, ChAT⁻ NK cell, and ChAT⁺ NK cell implantation; $P < 0.05$), without altering their distribution in the spleen ($29.50 \pm 2.78\%$, $30.75 \pm 2.66\%$, and $32.25 \pm 2.06\%$ for vehicle, ChAT⁻ NK cell, and ChAT⁺ NK cell implantation; $P > 0.05$) (Fig. 5C and D). The gating strategy for these immune cells was conducted as done previously (34).

Furthermore, we found that the loss of MBP in the spinal cords of EAE mice coincided with the large accumulation of CCR2⁺-infiltrating monocytes/macrophages, rather than the number of resident microglia that were GFP⁺ with distinct morphology in CX3CR1^{GFP/GFP}(CX3CR1^{-/-}) mice ($62.5 \pm 11.04/\text{mm}^2$ vs. $1518 \pm 70.19/\text{mm}^2$; $P < 0.05$) (Fig. 5E, F, and I). A better scenario ensued for ChAT⁻ NK cell-implanted mice, which underwent less severe demyelination with fewer CCR2⁺-infiltrating monocytes ($1518 \pm 70.19/\text{mm}^2$ vs. $1215.33 \pm 67.56/\text{mm}^2$; $P < 0.05$) (Fig. 5G and I). Interestingly, ChAT⁺ NK cell implantation into CX3CR1^{GFP/GFP} mice seemed to be efficacious in reducing the numbers of CCR2⁺-infiltrated monocytes/macrophages ($1215.33 \pm 67.56/\text{mm}^2$ vs. $664.5 \pm 55.69/\text{mm}^2$; $P < 0.05$) rather than the resident microglia, with the result that demyelination decreased (Fig. 5H and I).

The altered immune cell distribution within the CNS could be substantially affected by the cytokine microenvironment. RT-PCR conducted on the brain samples showed decreased expression of TNF- α , IL-1 β , IL-6, and IL-12 relative to the internal standard of hypoxanthine phosphoribosyltransferase 1 (HPRT-1) expression. Meanwhile, the change in IL-10 expression was negligible (Fig. 5J). These data indicate that the immune protective effect of ChAT⁺ NK cells in EAE was exerted by shaping the distribution of CCR2⁺ Ly6C^{hi} monocytes and the cytokine microenvironment in the CNS.

Reciprocal Chemoattraction Between ChAT⁺ NK Cells and CCR2⁺ Ly6C^{hi} Monocytes. To explore the underlying mechanisms that enable ChAT⁺ NK cells to reduce CCR2⁺Ly6C^{hi} monocyte content within the CNS, the communication between these cells was investigated. Immunofluorescent staining of the CD11b⁺CCR2⁺

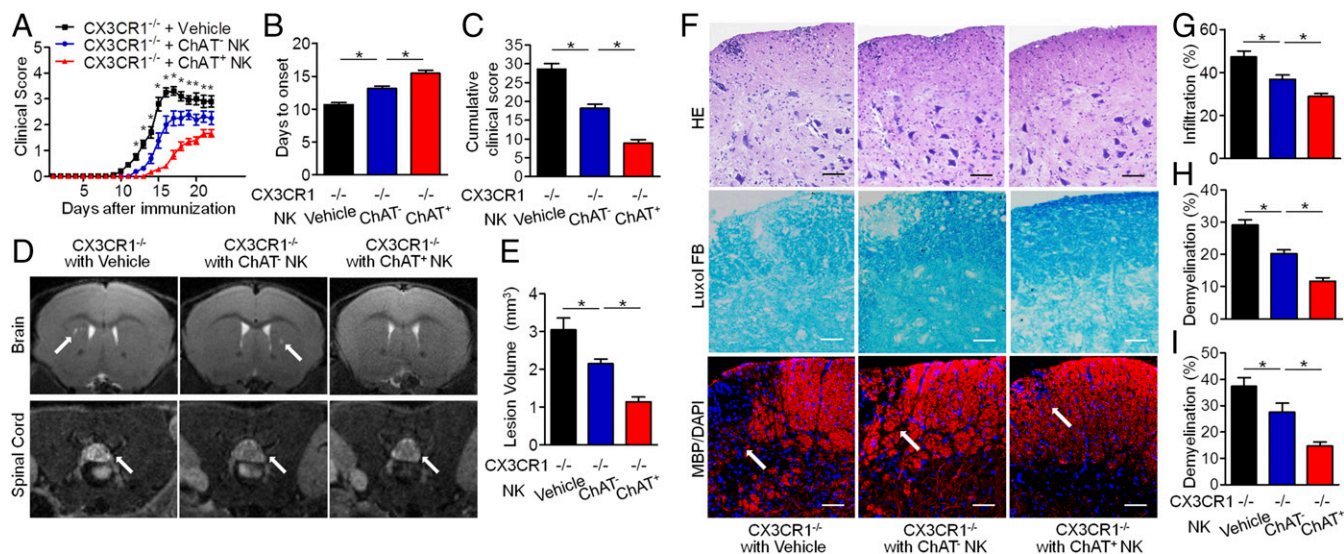


Fig. 4. CHAT⁺ NK cells have immune protective effects in the CNS of EAE mice. (A) Vehicle, ChAT⁻, or ChAT⁺ NK cells were implanted into cerebral ventricles of CX3CR1^{-/-} mice 10 d after EAE induction. The clinical scores of CX3CR1^{-/-} mice without treatment exceeded those implanted with NK cells from 12 to 22 dpi. ChAT⁺ NK cell implantation alleviated disease progression, as evident from lower clinical scores during the 12 to 22 dpi period. (B) The day of disease onset was earlier in CX3CR1^{-/-} mice and was significantly delayed by ChAT⁺ NK cell implantation. (C) The cumulative clinical score of CX3CR1^{-/-} mice was higher than that of CX3CR1^{-/-} mice with ChAT⁻ NK cells, and was significantly reduced by ChAT⁺ NK cell implantation. $n = 15$ per group for A–C. (D and E) T2-weighted images of brain and spinal cord samples from EAE mice were obtained by 7-T MRI. Arrows indicate lesions of increased signal intensity in the brain and spinal cord, which was reduced by ChAT⁺ NK treatment. (F–I) H&E staining shows immune cell infiltration into the spinal cord. LFB and MBP staining depict demyelination of the CNS along with large accumulations of immune cells in the lesion. Arrows indicate the demyelinated lesions by MBP staining. The statistics of demyelination reflected by Luxol FB (H) and MBP/DAPI (I) staining are shown. The implantation of ChAT⁺ NK cells reduced demyelination as well as decreasing the regional concentration of immune cells. $n = 10$ per group for D–I. Data are representative of six independent experiments. Mean \pm SEM. * $P < 0.05$. (Scale bars, 100 μ m).

monocytes and ChAT⁺ NK cells within the CNS showed the spatial proximity of these cells in EAE mice, which provided a favorable environment for the interaction between CCR2⁺Ly6C^{hi} monocytes and ChAT⁺ NK cells (Fig. 6A). To further ensure this occurrence, the chemoattractive effects of these cells were confirmed by transwell experiments. ChAT⁺ or ChAT⁻ NK cells were loaded into the upper chamber of the transwell system, and bone marrow-derived macrophages that were purified to yield a CCR2⁺Ly6C^{hi} population were loaded into the lower chamber. After 5 h of coculture, cells in the lower chamber were collected, and migrated NK cells were identified by FACS staining. The migratory ability of ChAT⁺ NK cells was much greater than that of ChAT⁻ NK cells (Fig. 6C). To investigate whether ChAT expression increased during migration or after encountering CCR2⁺Ly6C^{hi} monocytes, the ChAT expression of migrated NK cells was evaluated. Only a limited number of migrated NK cells originating from the ChAT⁻ upper chambers were found to become ChAT⁺ (Fig. 6B). The probable cause was the cytokine microenvironment or intercellular action with CCR2⁺Ly6C^{hi} monocytes, thereby facilitating NK cell expression of ChAT. In the transwell system, amounts of the cytokines IFN- γ , TNF- α , and GM-CSF expressed by migrated ChAT⁺ NK cells from the lower chamber were higher than that of the ChAT⁻ NK cells from the upper chamber according to RT-PCR detection, as was also seen for the expression of CCL3 and CCL4 (Fig. 6B and D). Meanwhile, mRNA expression profiling indicated that, compared with the other monocyte populations, CCR2⁺Ly6C^{hi} monocytes produced more of the chemokines CCL3, CCL4, CCL5, CXCL10, and CXCL11 as well as the cytokines IL-12, IL-15, and IL-18 (Fig. 6E and F). These results better elucidate the chemoattraction between ChAT⁺ NK cells and CCR2⁺Ly6C^{hi} monocytes, which communicate through the production of multiple cytokines and chemokines.

Immune Synapses Between ChAT⁺ NK Cells and CCR2⁺Ly6C^{hi} Monocytes Involve the NKG2A–Qa-1 Pathway and α 7-Nicotinic Acetylcholine Receptors. Based on the colocalization of CCR2⁺ infiltrative monocytes and ChAT⁺ NK cells in the CNS and the reciprocal

chemoattraction and communication between CCR2⁺Ly6C^{hi} monocytes and ChAT⁺ NK cells, we wondered whether the decrease of CCR2⁺Ly6C^{hi} monocytes in the CNS implanted with ChAT⁺ NK cells was executed via immune synapses formed between these cells and triggering the lytic activity or passing of an immune suppressive message. First, conjugate formation was quantified by FACS analysis, which indicated more synapse formation between ChAT⁺ NK cells and CCR2⁺Ly6C^{hi} monocytes than with ChAT⁻ NK cells (Fig. S1A–C). Immunological imaging analysis of the immune synapse showed the redistribution of α 7-nicotinic acetylcholine receptors (α 7nAChRs) on the surfaces of CCR2⁺Ly6C^{hi} monocytes accumulated toward the synapse with ChAT⁺ NK cells rather than evenly distributed on the surfaces of CCR2⁺Ly6C^{hi} monocytes alone (Fig. 7A). However, no polarization of α 7nAChRs in CCR2⁺Ly6C^{hi} monocytes was observed toward ChAT⁻ NK cells (Fig. S1D and F), thus indicating the potential involvement of these receptors in the communication within synapses. The decreased expression of TNF- α , IL-1 β , and IL-12 cytokines in CCR2⁺Ly6C^{hi} monocytes after coculture with ChAT⁺ NK cells (1.97 ± 0.18 vs. 1.17 ± 0.24 , 0.84 ± 0.04 vs. 0.43 ± 0.12 , and 1.25 ± 0.14 vs. 0.62 ± 0.16 relative expression for TNF- α , IL-1 β , and IL-12 after coculture with ChAT⁻ NK vs. ChAT⁺ NK cells, respectively; $P < 0.05$) was reversed in α 7nAChR knockout (KO) CCR2⁺Ly6C^{hi} monocytes (1.83 ± 0.13 vs. 1.99 ± 0.35 , 0.90 ± 0.19 vs. 0.80 ± 0.09 , and 1.40 ± 0.27 vs. 1.28 ± 0.35 relative expression for TNF- α , IL-1 β , and IL-12, respectively, after coculture with ChAT⁻ NK vs. ChAT⁺ NK cells; $P > 0.05$). This series of interactions indicates the important role of α 7nAChR in mediating this activity. After culturing with ChAT⁺ NK cells, the expression of IL-10 in CCR2⁺Ly6C^{hi} monocytes from WT (0.52 ± 0.09 vs. 0.52 ± 0.07 relative expression; $P > 0.05$) or α 7nAChR KO mice (0.52 ± 0.09 vs. 0.51 ± 0.17 relative expression; $P > 0.05$) did not change (Fig. 7C).

Perforin staining within the synapse also revealed the existence of cytotoxic activity during the intercellular action (Fig. 7B and Fig. S1E and G). To understand the mechanism that instigated

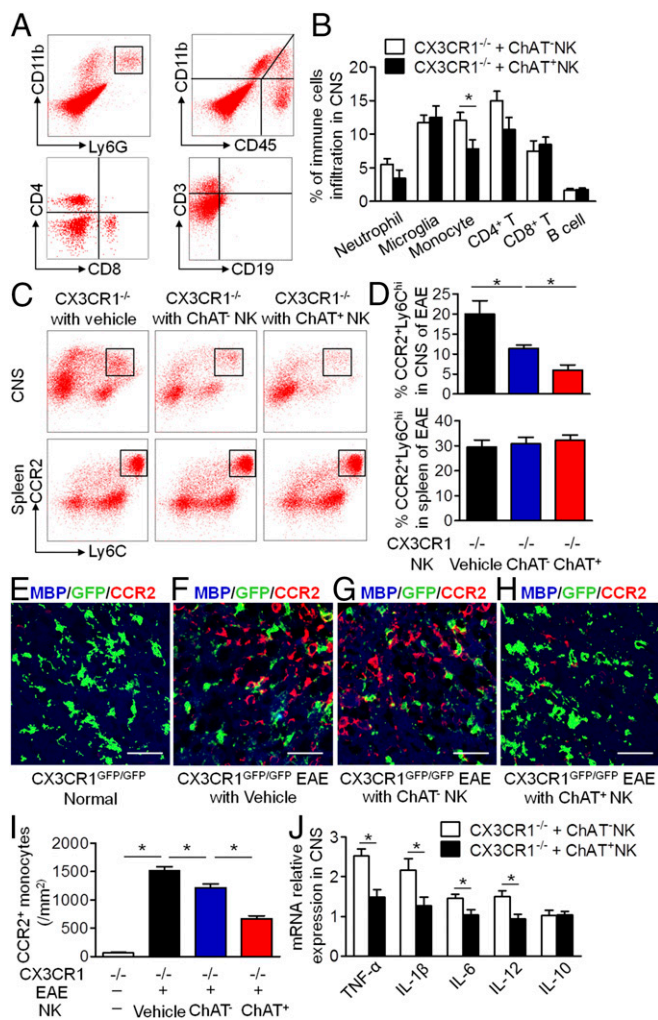


Fig. 5. ChAT⁺ NK cells influence the distribution of CCR2⁺Ly6C^{hi} monocytes and the cytokine microenvironment in the CNS of EAE mice. (A and B) The percentages of CNS-infiltrated neutrophils (CD11b⁺Ly6G⁺), CD8⁺ T cells, B cells (CD3⁺CD19⁺), and microglia (CD11b⁺CD45^{low}) were not changed by ChAT⁺ NK cell implantation. However, the numbers of infiltrated monocytes and CD4⁺ T cells ($P = 0.083$) decreased in ChAT⁺ NK cell-implanted groups. $n = 6$ per group. (C and D) The percentage of CCR2⁺Ly6C^{hi} monocytes in the CNS and spleen of CX3CR1^{-/-}, CX3CR1^{-/-} with ChAT⁻ NK cells, and CX3CR1^{-/-} mice with ChAT⁺ NK cells. ChAT⁺ NK cell implantation significantly decreased the numbers of CCR2⁺Ly6C^{hi} monocytes in the CNS without altering their distribution in the spleen. $n = 6$ per group. (E–H) MBP staining with CCR2 revealed a decrease of infiltrated CCR2⁺ monocytes/macrophages and reduced demyelination from ChAT⁺ NK cell treatment, without much effect on resident microglia (CX3CR1-GFP⁺). $n = 9$ per group. (I) Relative mRNA expression of TNF- α , IL-1 β , IL-6, IL-12, and IL-10 to HPRT-1 as the internal standard in CNS. $n = 6$ per group. Data are representative of three independent experiments. Mean \pm SEM. * $P < 0.05$. (Scale bars, 40 μ m).

the killing of autologous target cells by ChAT⁺ NK cells and mediated the antiinflammatory effect, further experiments were conducted. Coculturing CCR2⁺Ly6C^{hi} monocytes with ChAT⁺ NK cells resulted in their significantly reduced expression of Qa-1 compared with those cocultured with ChAT⁻ NK cells ($20.97 \pm 1.65\%$ vs. $15.53 \pm 1.59\%$; $P < 0.05$) (Fig. 7D and E). Meanwhile, similarly sorted NK cells were cocultured with CCR2⁺Ly6C^{hi} monocytes, after which the expression of ChAT-eGFP ($0.93 \pm 0.19\%$ vs. $2.33 \pm 0.27\%$; $P < 0.05$) and NKG2A ($15.43 \pm 1.33\%$ vs. $22.03 \pm 2.35\%$; $P < 0.05$) increased significantly. However, the expression of NKG2D in NK cells was not significantly different after the coculture ($0.30 \pm 0.12\%$ vs. $0.47 \pm 0.18\%$; $P > 0.05$) (Fig.

7F and G). The viability of CCR2⁺Ly6C^{hi} monocytes in the coculture system was then evaluated by 7-AAD staining (Fig. 7H). The susceptibility to killing by ChAT⁺ NK cells was reduced by Qa-1 up-regulation through lentivirus transfection, at effector:target ratios of 25:1, 10:1, and 5:1 ($28.20 \pm 1.39\%$ vs. $19.80 \pm 1.59\%$, $18.60 \pm 1.30\%$ vs. $14.37 \pm 1.18\%$, and $15.83 \pm 0.12\%$ vs. $12.33 \pm 1.02\%$ for 25:1, 10:1, and 5:1, respectively; $P < 0.05$). However, Qa-1 down-regulation exacerbated the likelihood of being killed by ChAT⁺ NK cells ($28.20 \pm 1.39\%$ vs. $33.57 \pm 1.73\%$, $18.60 \pm 1.30\%$ vs. $26.10 \pm 1.44\%$, and $15.83 \pm 0.12\%$ vs. $21.70 \pm 2.12\%$ for 25:1, 10:1, and 5:1, respectively; $P < 0.05$) (Fig. 7I).

For adoptive transfers to induce EAE, myelin oligodendrocyte glycoprotein (MOG)-responsive T cells and CCR2⁺Ly6C^{hi} monocytes were injected into Rag2^{-/-} γ c^{-/-} mice. ChAT⁺ NK cell implantation alleviated disease severity during EAE progression but had no effect on mice transferred with MOG-responsive T cells and α 7nAChR KO CCR2⁺Ly6C^{hi} monocytes (Fig. 7J). The foregoing results indicate that α 7nAChRs and the Qa-1–NKG2A pathway in the immune synapse between ChAT⁺ NK cells and CCR2⁺Ly6C^{hi} monocytes are important participants in the immune regulatory effect of ChAT⁺ NK cells toward CCR2⁺Ly6C^{hi} monocytes, through direct killing as well as modification of cytokine secretion.

Up-Regulation of Cholinergic Activity in NK Cells from MS Patients.

To determine the translational value of the foregoing observation to humans, the cholinergic system of NK cells from human peripheral blood was identified with determination of intracellular ACh production. NK cells were sorted from human peripheral blood samples by FACS to be used as a source of mRNA and protein extraction. RT-PCR and Western blot analysis of these human NK cells confirmed the expression of ChAT, VAcHT, AChE, and CHT1, which are essential components of the cholinergic system (Fig. 8A and B). Immunofluorescent staining of brain samples from MS patients also showed ChAT expression in infiltrated NK cells (NKp46⁺ChAT⁺GFAP⁻) (Fig. 8C). Staining of GFAP was involved to exclude astrocytes, which were reported to express NKp46 under an activated status in lesions of MS patients as well (42). Pathologic staining with MBP then revealed that those ChAT⁺ NK cells were preferentially located in the active demyelinated lesions of MS patients (Fig. 8C). Further, ACh production by NK cells from the peripheral blood was confirmed by UPLC-MS/MS detection. The intracellular concentration of ACh in NK cells from MS patients was higher than that from healthy individuals (0.1841 ± 0.00441 vs. 0.0187 ± 0.00561 ng/10⁶ cells; $P < 0.05$) (Fig. 8D). Additionally, the intracellular ACh concentrations showed a positive correlation with EDSS (Expanded Disability Status Scale) scores of the MS patients ($P < 0.05$) (Fig. 8E). The T2 high-intensity lesions detected in MRI scans from MS patients also revealed a positive correlation between ACh concentration and lesion volume ($P < 0.05$) or the multiplicity of the lesions ($P < 0.05$) (Fig. 8F–H). Overall, these data indicated that nonneural cholinergic activity of NK cells is up-regulated in CNS autoimmune disease and may be attributed to compensatory up-regulation in response to an overactivated autoimmune reaction in the MS setting. Therefore, the determination of ACh level in NK cells from the periphery could be an option for predicting the prognosis of patients with MS.

Discussion

Our results depict the antiinflammatory effect of ChAT⁺ NK cells in CNS inflammation involving the NKG2A–Qa-1 pathway and α 7nAChRs. Considering the widespread distribution of α 7nAChRs, ChAT⁺ immune cells could modulate the immune balance through the receptors especially under conditions of inflammatory disease (5, 6, 43). The expression and up-regulation of the nonneural cholinergic system are fine-tuned via chemical or neural stimulation as a mechanism to regulate or counteract the immune system (44,

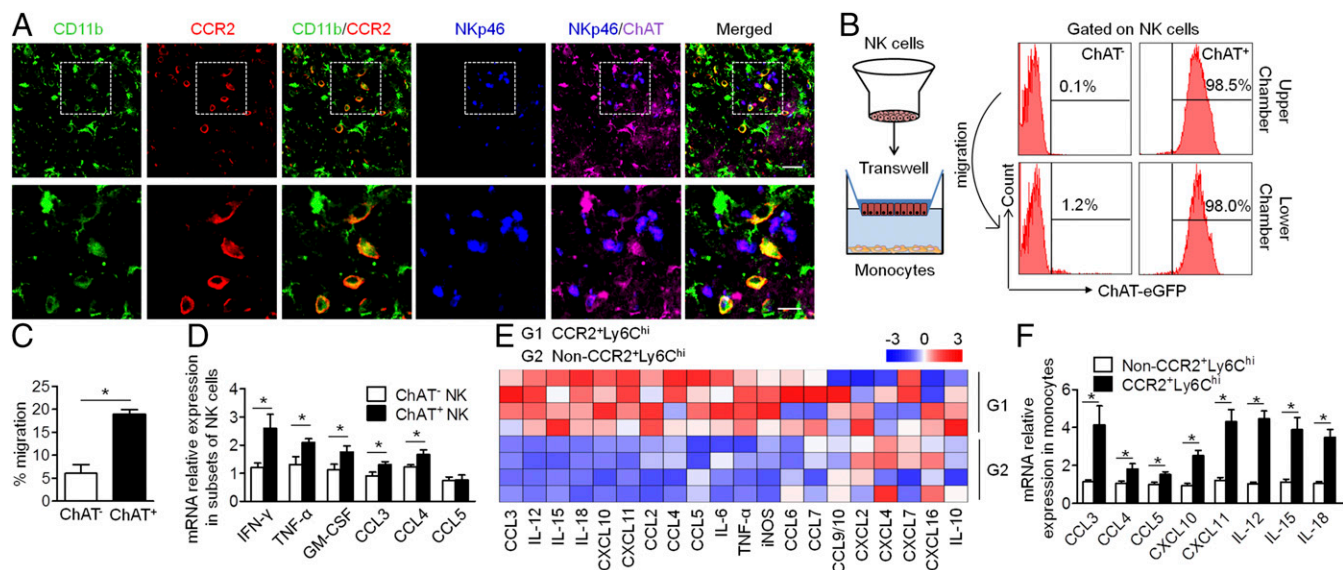


Fig. 6. ChAT⁺ NK cells and CCR2⁺Ly6C^{hi} monocytes are attracted reciprocally and communicate through various cytokines and chemokines. (A) ChAT⁺ NK cells and CD11b⁺CCR2⁺ infiltrative monocytes are colocalized in the CNS of EAE mice under confocal observation. Images are representative of three independent experiments. [Scale bars, 40 μm (Upper) and 20 μm (Lower).] (B and C) Sorted ChAT⁺ or ChAT⁻ NK cells were loaded into the upper chamber of the transwell system, and the lower chamber was loaded with bone marrow-derived monocytes. After 5 h of migration, the number of migrated NK cells was determined by FACS and is shown as a percentage of the total amount loaded. ChAT expression in the lower chamber that migrated from the ChAT⁺ or ChAT⁻ upper chamber was also evaluated. $n = 6$ per group from three independent experiments. (D) The relative mRNA expression of IFN- γ , TNF- α , GM-CSF, CCL3, CCL4, and CCL5 was detected in ChAT⁺ NK cells and ChAT⁻ NK cells. $n = 6$ per group of three independent experiments. (E) The mRNA expression of various cytokines/chemokines in CCR2⁺Ly6C^{hi} monocytes and non-CCR2⁺Ly6C^{hi} monocytes was detected. The expression level relative to the internal standard HPRT-1 appears in the heat map after Z transformation. (F) Among the genes detected, CCL3, CCL4, CCL5, CXCL10, CXCL11, IL-12, IL-15, and IL-18 were the most highly produced by CCR2⁺Ly6C^{hi} monocytes. $n = 6$ per group of three independent experiments. Mean \pm SEM. * $P < 0.05$.

45). Different from T or B cells, NK cells presumably respond quickly as part of innate immunity during inflammation. Therefore, an up-regulated expression of the cholinergic system in NK cells during the acute phase of inflammation could have a significant impact to restrict CNS inflammation.

NK cells develop functionally as they circulate through various organs after migrating from the bone marrow (46–49). Here, ChAT-eGFP expression was observed mostly in NK cell subsets of a mature phenotype in the periphery and was enhanced after EAE induction, which may correspond to the finding that more NK cells are mobilized under inflammatory conditions, possibly facilitating their maturation or activation (15, 50). However, the percentage of NK cells with ChAT expression did not seem to show a simple positive relationship with the severity of disease in certain organs. However, the acquisition and dynamic distribution of ChAT⁺ NK cells in the periphery and CNS along with disease progression reflected the close association between NK cell migration and ChAT expression. ChAT⁺ NK cells appear to possess greater migratory ability and are more readily recruited to sites of CNS injury. The more significant effect of ChAT⁺ vs. ChAT⁻ NK cells via the i.v. transfer to restrict CNS inflammation also supports this postulation (Fig. S2). Additionally, genome analysis depicted a distinctive expression profile of ChAT⁺ NK cells in terms of greater cytokine and chemokine production than their ChAT⁻ counterparts. Therefore, expression of the cholinergic system in NK cells is related to their propensity for CNS homing as well as phenotypic activation with regulatory functions.

Unlike the experiments with transgenic mice that selectively lack T or B cells for functional study in vivo, no mice are available that selectively lack NK cells (51–53). Consequently, we elected to use CX3CR1^{-/-} mice, because the loss of CX3CR1 on NK cells impairs the capability of NK cells to home in to the CNS. Functional analysis of ChAT⁺ NK cells after their transplantation into CX3CR1^{-/-} mice revealed a dramatic influence

on the distribution of CCR2⁺Ly6C^{hi} monocytes within the CNS, without much effect on microglia, CD8⁺ T cells, B cells, or neutrophils. The initiation and progression of EAE are associated with infiltration of immune cells into the CNS and subsequent immune responses between the adaptive and innate immune systems (54). CCR2⁺Ly6C^{hi} monocytes are among the earliest monocytes to infiltrate the CNS and are crucial for the development of EAE (55–58). Therefore, the decrease of CCR2⁺Ly6C^{hi} monocytes within the CNS after ChAT⁺ NK cell implantation appeared to contribute greatly to the lessening of EAE symptoms.

Unlike previous findings (18), the effect of these transplanted ChAT⁺ NK cells in the CNS was a preferential reduction of infiltrated monocytes/macrophages rather than resident microglia. The cause may have been chemotaxis between these cells and the timing of transplant intervention, during which the numbers of infiltrating monocytes exceeded that of the resident microglia. Even though the overall distribution of NK cells within the CNS of EAE mice revealed that ChAT⁺ NK cells were a small population (Fig. 2F and Fig. S3), the chemoattractive effect and intercellular communication seemed to “recruit” ChAT⁺ NK cells to accumulate around the CCR2⁺Ly6C^{hi} monocytes at inflammatory sites. The intercellular action is critical in shaping the immune activities of NK cells (19, 20, 22, 59), especially the activating effect of Ly6C^{hi} monocytes toward NK cells (30). Reciprocally, activated NK cells can influence the ability of proinflammatory monocytes to regulate immunity (21, 60, 61). In our experiments, ChAT⁺ NK cells reciprocally interacted with CCR2⁺Ly6C^{hi} monocytes through chemokine and cytokine secretion, thus regulating the immune functions of these cells.

NK cells are reported to engage in cross-talk with macrophages through various cytokines and are also contact-dependent within the immune synapse (60). The cytolytic effects toward target cells are based on the balance between the activated receptor NKG2D

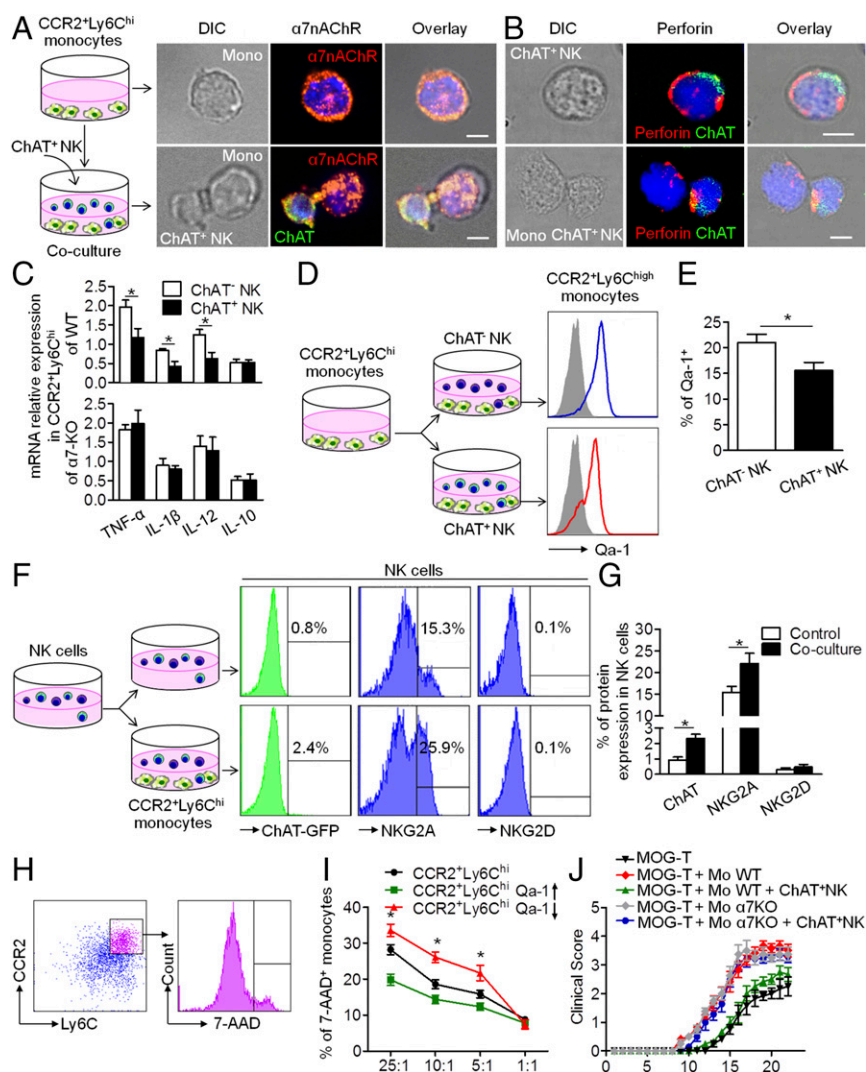


Fig. 7. Immune synapse between ChAT⁺ NK cells and CCR2⁺Ly6C^{hi} monocytes involved the NKG2A–Qa-1 pathway and α 7nAChR. (A) CCR2⁺Ly6C^{hi} monocytes were cocultured with ChAT⁺ NK cells. α 7nAChRs were evenly distributed on the surfaces of CCR2⁺Ly6C^{hi} monocytes alone but accumulated at the immune synapse with ChAT⁺ NK cells. Images represent three independent experiments. DIC, differential interference contrast. (Scale bars, 2 μ m.) (B) Cytotoxic granules containing perforin were polarized in ChAT⁺ NK cells toward CCR2⁺Ly6C^{hi} monocytes but evenly distributed when NK cells were cultured alone. Images represent three independent experiments. (Scale bars, 2 μ m.) (C) The expression of TNF- α , IL-1 β , and IL-12 in CCR2⁺Ly6C^{hi} monocytes from WT mice decreased after coculture with ChAT⁺ NK cells, whereas this expression remained unchanged in α 7nAChR KO CCR2⁺Ly6C^{hi} monocytes in the coculture system. There was no difference in IL-10 expression. $n = 6$ per group from three independent experiments. (D and E) Qa-1 expression on CCR2⁺Ly6C^{hi} monocytes was down-regulated after culture with ChAT⁺ NK cells. (F and G) NK cells were cocultured with CCR2⁺Ly6C^{hi} monocytes. Thereafter, the expression of ChAT-eGFP and NKG2A increased in NK cells; NKG2D remained consistent. $n = 6$ per group from three independent experiments for D–G. (H and I) The viability of CCR2⁺Ly6C^{hi} monocytes in the coculture system was evaluated by 7-AAD staining. Qa-1 expression up-regulated by lentivirus transfection in CCR2⁺Ly6C^{hi} monocytes rendered them less susceptible to killing by NK cells, whereas down-regulation exacerbated the killing. $n = 6$ per group from two independent experiments. (J) ChAT⁺ NK cell implantation alleviated symptoms reflected in high clinical scores of EAE mice administered MOG-responsive T cells and CCR2⁺Ly6C^{hi} monocytes of WT, but had no effect on EAE mice given α 7nAChR KO CCR2⁺Ly6C^{hi} monocytes. $n = 9$ per group from three independent experiments. Mean \pm SEM. * $P < 0.05$.

and the inhibitory receptor NKG2A (62–64). Results from our microassays indicated that the ChAT⁺ NK cells had no increase of cytolytic activity, which was confirmed *in vitro* by a cytotoxic assay of YAC-1 cells (Fig. S4A and B). However, the specific lysis of autologous CCR2⁺Ly6C^{hi} monocytes in coculture with ChAT⁺ NK cells was higher than that with the ChAT[−] subset (Fig. S4A and B). Actually, this “cytotoxicity” toward autologous immune cells was largely due to the lack of Qa-1 on CCR2⁺Ly6C^{hi} monocytes cultured with ChAT⁺ NK cells, because Qa-1 reduction broke down the immune tolerance of those cells under the surveillance of autologous NK cells, instead of the cytotoxicity of the NK cells themselves (Fig. 7D). Besides, the ligands for NKG2D, RAE-1, and MULT-1 on CCR2⁺Ly6C^{hi} monocytes were not altered significantly after culture with ChAT⁺ NK cells (Fig. S4C), indicating how important the NKG2A–Qa-1 pathway may be as a mediator of this activity. The ligands expressed in CCR2⁺Ly6C^{hi} monocytes of the CNS by ChAT⁺ NK cell implantation also represented the down-regulation of Qa-1 *in vivo* (Fig. S4D and E), although the reason for that down-regulation remains unclear and in need of inquiry. The redistribution of α 7nAChRs accumulated in the immune synapse might constitute a basic structure for the cholinergic antiinflammatory effect of locally concentrated ACh derived from ChAT⁺ NK cells. The down-regulation of Qa-1 in CCR2⁺Ly6C^{hi} monocytes was observed upon nicotine treatment in culture or in adoptively transferred

EAE with ChAT⁺ NK cell implantation, activity that was reversed by α 7nAChR KO (Fig. S4F and G), thus suggesting involvement of the cholinergic effect as well as α 7nAChRs in regulating Qa-1 expression.

The up-regulation of ChAT expression under abnormal autoimmune conditions was verified in peripheral NK cells from MS patients (Fig. S5A–C). Further experiments confirmed that these changes resulted mainly from the up-regulation of ChAT expression in CD56^{bright} subsets of NK cells (Fig. S5D–F), which were reported to be activated under MS conditions rather than the CD56^{dim}CD16^{bright} NK cells (15). Unlike murine NK cells, human NK subsets did not show a simple increase in ChAT expression along with their maturation from CD56^{bright} to CD16^{bright} NK cells in normal conditions; an up-regulation in CD56^{bright} subsets was found only in MS patients. Therefore, ACh synthesis seems to be largely affected by inflammatory circumstances, and the functional response occurs along with mobilization of this subset of NK cells. CD56^{bright} NK cells have been known to play important roles in MS, including a heightened capacity for migration from the periphery into the CNS and accumulation in the demyelinated lesions where they interact with local immune cells (15). Importantly, CD56^{bright} NK cells in the peripheral circulation were found to undergo MS-related activation, although the CD56^{dim}CD16^{bright} subpopulation decreased (15). This cholinergic up-regulation could be induced by

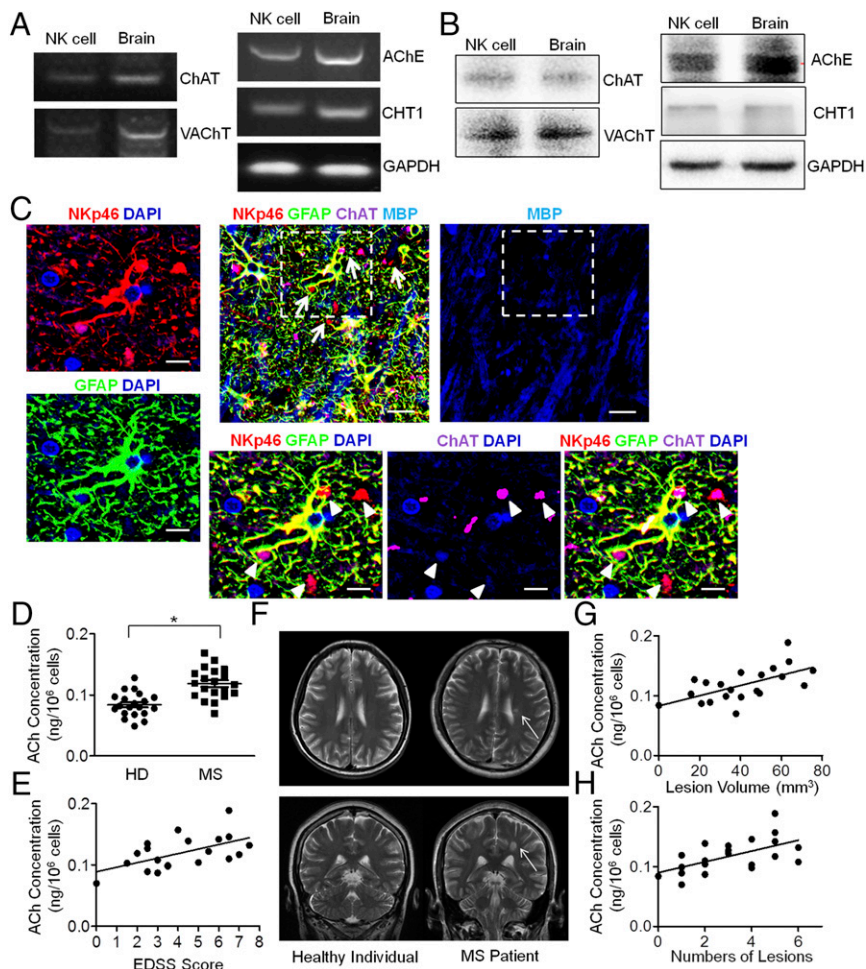


Fig. 8. Cholinergic system of human NK cells and the up-regulation of ACh synthesis in MS patients. (A and B) Components of the cholinergic system (ChAT, VAcHT, AChE, CHT1) were identified in human NK cells by RT-PCR and Western blot, compared with the positive control for brain samples. PCR bands: ChAT, 403 bp; VAcHT, 284 bp; AChE, 163 bp; CHT1, 385 bp; GAPDH, 120 bp; Western blot bands: ChAT, 68 kDa; VAcHT, 72 kDa; AChE, 68 kDa; CHT1, 51 kDa; GAPDH, 36 kDa. $n = 5$. (C) Cells with NKp46 expression (Upper Middle) were found mostly in areas with demyelination of white matter, as reflected by the MBP staining (Upper Right). White matter lesions infiltrating NK cells were identified as NKp46⁺GFAP⁺, as the arrows indicate (Upper Middle). (Scale bars, 20 μ m.) Images with higher magnification of the dashed box indicated area are shown to depict the NK cells (NKp46⁺GFAP⁺) (arrowheads) (Lower Left) with ChAT expression (Lower Right). (Scale bars, 8 μ m.) (D) UPLC-MS/MS detection of intracellular ACh production from human NK cells showed higher levels in MS patients compared with healthy individuals (HD). $n = 20$ per group. (E) The intracellular ACh concentrations from NK cells of MS patients also correlated with the EDSS scores. $n = 20$ per group. $P < 0.05$. (F) Images of axial and coronal T2 MRIs showed hyperintensity of the demyelinated lesions around the peripheral ventricles. Representative images from $n = 20$ per group. (G and H) MRI scanning showed a positive correlation between intracellular ACh concentration and lesion volume ($P < 0.05$) or numbers of lesions ($P < 0.05$). $n = 20$ per group. Mean \pm SEM. * $P < 0.05$.

the overactivated inflammatory environment, which in turn might exert immunosuppressive effects to counteract the internal immune responses. Potentially, determining intracellular ACh concentrations in such patients could provide a prognostic method for estimating the severity of disease and offer a new strategy for MS intervention.

Materials and Methods

Detailed information on materials and methods used in this study is provided in *SI Materials and Methods*.

Mice. C57BL/6 WT mice were purchased from Vital River. ChAT-eGFP, CX3CR1^{GFP/GFP} (CX3CR1^{-/-}), and $\alpha 7$ nAChR KO mice were purchased from the Jackson Laboratory. Rag2^{-/-} γ c^{-/-} mice were purchased from Taconic Biosciences. EAE and adoptively transferred EAE were induced in these animals as described. The experiments were reviewed and approved by the animal care and use committees of Tianjin Medical University and Tianjin Neurological Institute.

FACS Analysis and FACS Sorting. Isolated immune cells were assessed and sorted according to standard protocols.

PCR. cDNA was prepared from sorted cells or indicated tissues, and RT-PCR was performed with the outer primers (Table S1). For quantitative determination, qRT-PCR was performed with template and primers as displayed in Table S2.

UPLC-MS/MS. Isolated NK cells were homogenized and deproteinized. Supernatants with an internal standard (d_5 -ACh; CDN) added were obtained for UPLC-MS/MS analysis. The conditions of the system were adjusted following the parameters as published (36).

Cellular Transplantation. A total volume of 10 μ L NK cells suspended at a concentration of 5×10^6 per mL was loaded into a 10- μ L Hamilton syringe. The amount of NK cells to be implanted into the CNS was determined by dose-dependent quantification (Fig. S6). EAE was induced in CX3CR1^{-/-} mice, with cells or vehicles injected into their lateral cerebral ventricles at ~ 10 to 13 dpi.

Neuropathology and Neuroimaging. Samples were prepared for H&E (Solarbio), LFB, and immunofluorescent staining. MRI was performed using a 7-T small-animal MRI instrument for acquiring T2-weighted images.

Cell Culture and Migratory, Conjugative, and Viability Assays. After isolation, cells were cultured under the indicated conditions for migration, conjugation (65–67), lentivirus transfection, and viability assays as described.

Human Subjects and Quantification of NK Cell-Derived ACh. Brain sections from patients with relapsing–remitting MS were acquired from the Sun Health Research Institute and St. Joseph's Hospital and examined retrospectively. The protocols and informed consent were approved by the Institutional Review Board of the Banner Sun Health Institute and St. Joseph's Hospital. Patients or their caregivers provided informed consent for brain donation as well as for the purpose of research analysis. Thirty-eight patients with relapsing–remitting MS at the acute stage of the disease were enrolled in this study (Table S3). The protocol was approved by Tianjin Medical University General Hospital, and informed consent was obtained from all patients at enrollment.

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