

Transcriptome and Function of Novel Immunosuppressive Autoreactive Invariant Natural Killer T Cells That Are Absent in Progressive Multiple Sclerosis

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Abstract

Background and Objective

The aim of this study was to determine whether natural killer T (NKT) cells, including invariant (i) NKT cells, have clinical value in preventing the progression of multiple sclerosis (MS) by examining the mechanisms by which a distinct self-peptide induces a novel, protective invariant natural killer T cell (iNKT cell) subset.

Methods

We performed a transcriptomic and functional analysis of iNKT cells that were reactive to a human collagen type II self-peptide, hCII707-721, measuring differentially induced genes, cytokines, and suppressive capacity.

Results

We report the first transcriptomic profile of human conventional vs novel hCII707-721-reactive iNKT cells. We determined that hCII707-721 induces protective iNKT cells that are found in the blood of healthy individuals but not progressive patients with MS (PMS). By transcriptomic analysis, we observed that hCII707-721 promotes their development and proliferation, favoring the splicing of full-length AKT serine/threonine kinase 1 (AKT1) and effector function of this unique lineage by upregulating tumor necrosis factor (TNF)-related genes. Furthermore, hCII707-721-reactive iNKT cells did not upregulate interferon (IFN)- γ , interleukin (IL)-4, IL-10, IL-13, or IL-17 by RNA-seq or at the protein level, unlike the response to the glycolipid alpha-galactosylceramide. hCII707-721-reactive iNKT cells increased TNF α only at the protein level and suppressed autologous-activated T cells through FAS-FAS ligand (FAS-FASL) and TNF α -TNF receptor I signaling but not TNF receptor II.

Discussion

Based on their immunomodulatory properties, NKT cells have a potential value in the treatment of autoimmune diseases, such as MS. These significant findings suggest that endogenous peptide ligands can be used to expand iNKT cells, without causing a cytokine storm, constituting a potential immunotherapy for autoimmune conditions, including PMS.

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Glossary

AKT1 = AKT serine/threonine kinase 1; **cDMEM** = complete Dulbecco's Modified Eagle Medium; **CFSE** = carboxyfluorescein succinimidyl ester; **convT** = conventional CD4+ T cells; **DMEM** = Dulbecco's Modified Eagle Medium; **DN** = double-negative; **EAE** = experimental autoimmune encephalomyelitis; **FACS** = fluorescence-activated cell sorting; **FAS-FASL** = FAS-FAS ligand; **FDR** = false discovery rate; **GSEA** = gene set enrichment analysis; **HC** = healthy control; **HD** = healthy donor; **IFN** = interferon; **IL** = interleukin; **iNKT cell** = invariant natural killer T cell; **MS** = multiple sclerosis; **NKT** = natural killer T cell; **PMS** = progressive MS, including PPMS and SPMS; **PBMC** = peripheral blood mononuclear cell; **PPMS** = primary progressive MS; **RRMS** = relapsing-remitting MS; **SPMS** = secondary progressive MS; **TNF** = tumor necrosis factor; **TNFR1** = TNF receptor I; **TNFR2** = TNF receptor II; **α -GalCer** = alpha-galactosylceramide; **7AAD** = 7-aminoactinomycin D.

Natural killer T (NKT) cells recognize lipid and glycolipid antigens on the major histocompatibility complex Class I-related CD1d.¹⁻⁵ In addition to their host defense⁶ and anti-tumor activities,⁷ NKT cells are linked to the prevention of inflammation in allergic and autoimmune diseases.⁸⁻¹⁰ Multiple sclerosis (MS) is a chronic neuroinflammatory disease that is believed to be mediated by autoreactive lymphocytes, implicating a defect in NKT cells.^{11,12} Invariant iNKT (iNKT) cell levels are lower in the peripheral blood of patients with MS,^{13,14} and untreated patients with MS have more Th2-skewed iNKT cells during remission.¹⁵ iNKT cells can be induced by the glycolipid alpha-galactosylceramide (α -GalCer) to modulate experimental autoimmune encephalomyelitis (EAE), a model of MS, through Th2 cytokines.^{16,17} However, α -GalCer treatment for EAE in mice causes liver toxicity,¹⁸ induces abortions,¹⁹ and exacerbates atherogenesis²⁰—possibly from a cytokine storm. Consistent with this model, on induction with α -GalCer, NKT cells secrete high levels of IFN- γ and IL-4, which can be harmful in MS.¹¹ Thus, NKT cell responses to antigen must be discerned to develop NKT cell-based therapeutics. We have found that self-antigenic peptide-reactive NKT cells maintain peripheral tolerance, demonstrating that an immunodominant epitope from mouse collagen type II (mCII707-721) induces NKT cells, in turn inhibiting arthritis, neuroinflammation, and lung inflammation in models of rheumatoid arthritis, MS, and allergic asthma, respectively.²¹

We report a novel self-peptide-reactive NKT cell population in human peripheral blood. On being challenged with hCII707-721, they are activated and immunosuppressive in healthy individuals but hyporesponsive in patients with MS. Furthermore, we present the first transcriptional profile of human iNKTs, based on the effects of hCII707-721.

Methods

Peripheral Blood Samples

Peripheral blood was collected by standard venipuncture into vacuum tubes with ethylenediaminetetraacetic acid. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-isopaque density gradient centrifugation (Gibco-BRL). Peripheral blood from untreated relapsing-remitting MS (RRMS) and progressive MS (PMS) (primary progressive MS [PPMS])

and secondary progressive MS [SPMS]) patients, classified per Lublin and Reingold,⁴⁸ was obtained from the Danish Multiple Sclerosis Center and Multiple Sclerosis Center of Catalonia; blood from healthy donors (HDs) was acquired from Rigshospitalet, Denmark.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Scientific Ethics Committee for Copenhagen and Frederiksberg (protocol KF01-041/95) and the Hospital Universitari Vall d'Hebron Ethics Committee [PR(AG)32/2008] and was conducted after individual consent was obtained.

Patients and Healthy Individuals

In this cross-sectional study, in total, 146 subjects were included: 44 age- and gender-matched healthy controls (HCs), 26 patients with RRMS, 34 patients with PPMS, and 42 patients with SPMS. eTable 6 (links.lww.com/NXI/A555) details all the individuals' information included age, EDSS score, and disease duration.

Baseline CD4⁺ and CD8⁺ double-negative (DN) NKT cells were studied in patients with MS and HCs who had been recruited to the Danish MS Center, comprising HC: 24 women (mean age 37 years; range 24–62) and 8 men (39 years; 24–63), patients with RRMS: 14 women (38 years; 21–55) and 5 men (34 years; 28–41), PPMS: 8 women (51 years; 43–59) and 12 men (47 years; 28–59), and SPMS: 11 women (46 years; 33–60) and 9 men (46 years; 30–53).

Baseline iNKT cells were analyzed in patients with MS and HDs from the Danish MS Center; the blood bank at Rigshospitalet, Denmark; and Universitari Vall d'Hebron, Spain. The study population comprised HD: 9 women (44 years; 26–58) and 3 men (30 years; 23–37), patients with RRMS: 4 women (30 years; 24–37) and 2 men (38 years; 36–41 years), PPMS: 6 women (55 years; 52–60) and 6 men (51 years; 31–62), and SPMS: 7 women (54 years; 53–64) and 8 men (50 years; 36–62).

The responsiveness to hCII707-721 was tested in vitro in freshly isolated human PBMCs from patients with PMS from the Danish MS Center and HDs. The 39 PBMC samples comprised 12 HDs, 12 PPMS, and 15 SPMS—HDs: 9 women

Table 1 Demography of Individual Subjects Included in the Study

Flow cytometry study	Healthy subject or MS	Gender and number of samples	Mean and range in years	Clinic
Baseline DN NKT	HC	Female n = 24	37 (24–62)	Danish MS Clinic
		Male n = 8	39 (24–63)	Danish MS Clinic
	RRMS	Female n = 14	38 (21–55)	Danish MS Clinic
		Male n = 5	34 (28–41)	Danish MS Clinic
	PPMS	Female n = 8	51 (43–59)	Danish MS Clinic
		Male n = 12	47 (28–59)	Danish MS Clinic
	SPMS	Female n = 11	46 (33–60)	Danish MS Clinic
		Male n = 9	46 (30–53)	Danish MS Clinic
Baseline iNKT	HD	Female n = 9	44 (26–58)	Blood Bank
		Male n = 3	30 (23–37)	Blood Bank
	RRMS	Female n = 4	30 (24–37)	Universitari Vall d'Hebron MS Clinic
		Male n = 2	38 (36, 41)	Universitari Vall d'Hebron MS clinic
	PPMS	Female n = 6	55 (52–60)	Danish MS Clinic
		Male n = 6	51 (31–62)	Danish MS Clinic
	SPMS	Female n = 7	54 (53–64)	Danish MS Clinic
		Male n = 8	50 (36–62)	Danish MS Clinic
In vitro hCII707-721 Reactivity	HD	Female n = 9	44 (26–58)	Blood Bank
		Male n = 3	30 (23–37)	Blood Bank
	PPMS	Female n = 6	55 (52–60)	Danish MS Clinic
		Male n = 6	51 (31–62)	Danish MS Clinic
	SPMS	Female n = 8	54 (36–64)	Danish MS Clinic
		Male n = 7	50 (36–62)	Danish MS Clinic
iNKT Suppressive assay and phenotyping	HD	Female n = 13	33 (19–57)	Blood bank
		Male n = 11	42 (21–59)	Blood bank
RNA-seq	HD	Female n = 3	30–40	Blood bank

Abbreviations: DN = double-negative; HC = healthy control; HD = healthy donor; iNKT = invariant NKT; MS = multiple sclerosis; NKT = natural killer; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SPMS = secondary progressive MS.

(44 years; 26–58) and 3 men (30 years; 23–37 years), PPMS: 6 women (55 years; 52–60) and 6 men (51 years; 31–62), and SPMS: 8 women (54 years; 36–64) and 7 men (50 years; 36–62).

In vitro phenotyping and suppression of iNKT cells were analyzed in 24 HDs: 13 women (33 years; 19–57) and 11 men (42 years; 21–59). Table 1 details the patients and HC included in the various assays.

None of the patients have received immunosuppressive or immunomodulatory treatment before sample date.

Preparation and Treatment of Human PBMcs

Buffy coats of blood donors and blood (15 mL) from patients with MS (10–12 mL) were used to prepare PBMcs using the Ficoll-Paque Plus (7.5 mL) (GE Healthcare, 17-1440-02). The cells were then centrifuged at 400g for 30–40 minutes at 18°C–20°C. The lymphocyte layer was collected, washed 3 times with PBS, and centrifuged at 300g for 10 minutes. The cells were resuspended at 3×10^6 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax-1 (Gibco), 10% fetal bovine serum (Sigma), 1% HEPES 1 M (Invitrogen), 1% penicillin-streptomycin 10,000 U/mL (Gibco), and 0.001% beta-mercaptoethanol 50 mM to make complete DMEM

(cDMEM). PBMCs were analyzed or stored in liquid nitrogen until use.

Human Collagen II Peptide 707-721-Activated NKT Cells

PBMCs were cultured in round-bottomed 96-well culture plates (Nunc) in a humidified 37°C atmosphere, containing 5% CO₂. PBMCs were treated with 5 µg/mL α-GalCer (KRN7000 Funakoshi Co, Ltd.), 100 µg/mL hCII707-721 (H-PPGSNGNPGPPGPPG-OH Schafer-N), or collagen type I 707-721 as a negative control. Cells were stained with antibodies after 48 hours for a fluorescence-activated cell sorting (FACS) analysis.

FACS Staining

PBMCs were washed with PBS and 0.5% BSA (Sigma). Fc receptors (FcR) were blocked with antihuman FcR blocking reagent (Miltenyi Biotec 130-059-901) in PBS and 2% BSA for 30 minutes at 4°C per the manufacturer's instructions. All antibodies were used at 1–5 µg/mL: TCR αβ anti-human Pacific Blue (IP26 306716 Biolegend), CD4 antihuman APC-Cy7 (RPA-T4 300518 Biolegend), CD8 antihuman BV510 (RPA-T8 563526 BD Biosciences), CD56 antihuman BV605 (HCD56 318334 Biolegend), CD161 antihuman FITC (191B8 130-092-097 Miltenyi Biotec), CD94 antihuman APC (HP-3D9 17-5094 eBioscience) or Vα24Jα18 TCR antihuman APC (6B11 17-5806 eBioscience), CD178 (FasL) antihuman PE (NOK-1 306407 Biolegend), CD3 antihuman APC-Cy7 (HIT3a 300318 Biolegend), CD4 Pe-Cy7 antihuman (RPA-T4 300512 Biolegend), CD8 antihuman eFluorNC 605 (RPA-T8 94-0088 eBioscience), CD56 antihuman PerCP-Cy5.5 (HCD56 318322 Biolegend), and CD161 antihuman APC (DX12 V1NK12 BD Biosciences). 7-Aminoactinomycin D (7AAD) (BD Pharmingen 559925) was applied to exclude dead cells.

Intracellular cytokine staining was performed in iNKT magnetic bead-enriched PBMCs after blockade with Brefeldin-A 10 µg/mL (420601 Biolegend) for 6 hours, using IL-4 antihuman PE (8D4-8 500704 Biolegend), IFN-γ antihuman Alexa Fluor-700 (4S.B3 502520 Biolegend), TNFα antihuman Alexa Fluor-488 (Mab11 55,722 BD Pharmingen), IL17F antihuman PerCP efluor- 710 (SHLR17 46-7169 eBioscience), granzyme B antihuman/mouse FITC (GB11 515403 Biolegend), perforin antihuman APC (DG9 308111 Biolegend), and TGF-β1 antihuman PE-Cy7 (TW4-2F8 349610 Biolegend).

Cells were acquired on a BD LSR II using FACSDiva and analyzed in FlowJo (Tree), v8.8.7.

Purification of iNKT Cells

PBMCs (4–10 × 10⁸) were cultured in cDMEM at 3 × 10⁶ cells/mL with hCII707-721 (100 µg/mL) in T-175 cm² flasks (Nunc/Fischer). After activation with hCII707-721 for 48 hours, total iNKT cells were purified by autoMACS using Anti-iNKT MicroBeads (130-094-842, Miltenyi Biotec). To

distinguish the hCII707-721-treated iNKT cells before purification from nontreated iNKT cells, the hCII707-721-treated iNKT cell population is referred to as hCII707-721-reactive iNKT cells.

CFSE Staining

PBMCs were labeled with fresh 1 µM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) at 37°C for 2 minutes and then at room temperature for 7 minutes in the dark. An equal volume of fetal bovine serum was added to halt the reaction, and the cells were washed 3 times with cDMEM.

In Vitro Suppression Assay

PBMCs were labeled with CFSE and activated (2 × 10⁵ cells/well) with plate-bound anti-CD3 10 µg/mL (OKT3 16-003781 eBioscience) and human soluble anti-CD28 2 µg/mL (CD28.2 16-028981 eBioscience) for 48 hours. Autologous hCII707-721-reactive iNKT cells from the same HD were purified by autoMACS and cocultured at 1:1 with CFSE⁺ PBMCs for another 24 hours.

For certain experiments, 1 hour before coculture, PBMCs were blocked with antibodies against human Fas (250 ng/mL, ZB4 Millipore 05-338), TNF receptor I (TNFRI) (10 µg/mL, H398 In vivo Gen mab-htnfr1), and TNF receptor II (TNFRII) (0.5 µg/mL, 22221 R&D Systems MAB226). IgG (BD Pharmingen Purified Mouse IgG/IgG2a κ Isotype 564121/554126) was used as a negative control.

After 24 hours of coculture, cells were harvested and supplemented with 7ADD before FACS analysis of CFSE⁺ vs 7AAD⁺ cells.

ELISA

Supernatants from in vitro PBMC cultures, treated with α-GalCer and hCII707-721 for 48 hours, were analyzed using the Granzyme B Platinum ELISA kit (Affymetrix eBioscience BMS2027) per the manufacturer's instructions.

RNA-Seq Sample Preparation and Gene Expression Analysis

Total RNA was extracted from PBMCs samples (3 women HDs, 30–40 years) using the RNeasy mini kit (Qiagen, 74,104). The RNA-seq samples were composed of 3 biological individual samples for each population, that is, 3 conventional T cells, 3 conventional iNKT cells (without hCII707-721 activation), and 3 hCII707-721-reactive iNKT cells (with hCII707-721 activation). Messenger ribonucleic acid was prepared using oligo-dT primers and reverse transcribed (KAPA Biosystems, KK2601). The double-stranded DNA was tagged (Illumina, FC-131-1096) and ligated with sequencing adaptors (Illumina, FC-131-2002) as detailed.⁴⁹

Libraries were pooled (1.6 pM) and sequenced through paired-end 75-bp reads on an Illumina NextSeq500. Three biological replicates were used for RNA-seq for each condition (3 healthy women, 30–40 years). Quality control and

preprocessing steps were performed using FastQC and Trimomatic. Gene expression was quantified by mapping the reads to the human genome (GRCh38.p13, GENCODE v32) using Salmon (v0.14.1). On average, there were 10.5 M mapped reads per library. Pairwise comparison of biological replicates between conditions was performed using edgeR. Differential isoform usage was analyzed using IsoformSwitchAnalyzer.⁵⁰ Pathway and gene ontology enrichment analysis was performed in R using ReactomePA,⁵¹ CAMERA,⁵² and fgsea.⁵³⁻⁵⁵ For the pathway enrichment analysis, we used annotated gene sets from the Molecular Signatures Database.⁵⁴

Data Availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Results

Reduced Circulating NKT Cells and a Lack of hCII707-721-Reactive iNKT Cells in PMS

Patients with PMS are often older than patients with RRMS, and because age could be a confounding factor affecting immune cells, we investigated first if age affects the frequency of NKT cells. The correlation analysis of age vs subset frequency in the HCs did not reveal a significant impact of the age interval used in the study on the frequency of NKT cells (eFigure 1, links.lww.com/NXI/A547).

In examining whether PMS is associated with NKT subsets using established markers, we did not find any differences in single positive CD3⁺ or CD4⁺ NKT cells between MS and HCs, nor did we find differences based on CD56⁺, CD95⁺, and CD161⁺ triple-positive population (data not shown). However, in support of previous studies,^{13,14} among double-negative cells (CD4⁻CD8⁻DN²²), the percentages of CD4⁻CD8⁻CD161⁺ NKT cells and CD4⁻CD8⁻CD56⁺CD161⁺ NKT cells were lower in patients with PPMS (Figure 1A–C). Based on the invariant TCR α -chain Va24Ja18 (Figure 1D), we found a decrease in iNKT cells, albeit insignificant, in both PMS patient groups but not RRMS patients (Figure 1E).

Healthy individuals harbored no hCII707-721-reactive CD4⁺ or DN NKT cells after a 48-hour challenge with this peptide (not shown) but had a significant population of a novel hCII707-721-reactive iNKT subset (Figure 1F,G). Gating on Va24Ja18⁺ iNKT cells (Figure 1F), hCII707-721, not α -GalCer, significantly increased the percentage of iNKTs in HDs (Figure 1G). Furthermore, patients with PMS, particularly those with SPMS, had fewer hCII707-721-reactive iNKT cells vs HDs (Figure 1G).

Human iNKT-specific Transcriptomic Signature

No complete gene signature of human iNKT cells has been reported. To address this shortcoming, we purified naïve conventional CD4⁺ T cells (convT), conventional iNKT cells, and hCII707-721-reactive iNKT cells from PBMCs and Va24Ja18-expressing cells, respectively, and performed RNA sequencing. We considered all iNKTs as an entity, regardless of their

activation status, based on their clustering in principal component analysis plots and gene expression profiles (false discovery rate [FDR] < 0.05) (Figure 2A and eFigure 2A and B, links.lww.com/NXI/A548).

iNKT cells had a distinct transcriptional profile compared with convT cells, wherein 1927 genes were differentially expressed (FDR < 0.05) (Figure 2A–B, eTable 1, links.lww.com/NXI/A550), 139 of which were lncRNAs (Figure 2C, eTable 2, links.lww.com/NXI/A551). Of these 1,927 genes, 1,397 were significantly upregulated and 530 were downregulated (Figure 2B, D). Next, we annotated cell surface markers and transcription factors²³ among these differential genes to identify the top surface markers and a transcription factor network that were related to iNKT function—constituting the gene signature of iNKTs (Figure 2E–F, eTable 1). The iNKT-specific transcription factor profile was used to perform STRING network analysis,²⁴ in which overrepresented Reactome pathways were identified among the core factors (eFigure 2A–C, links.lww.com/NXI/A548).

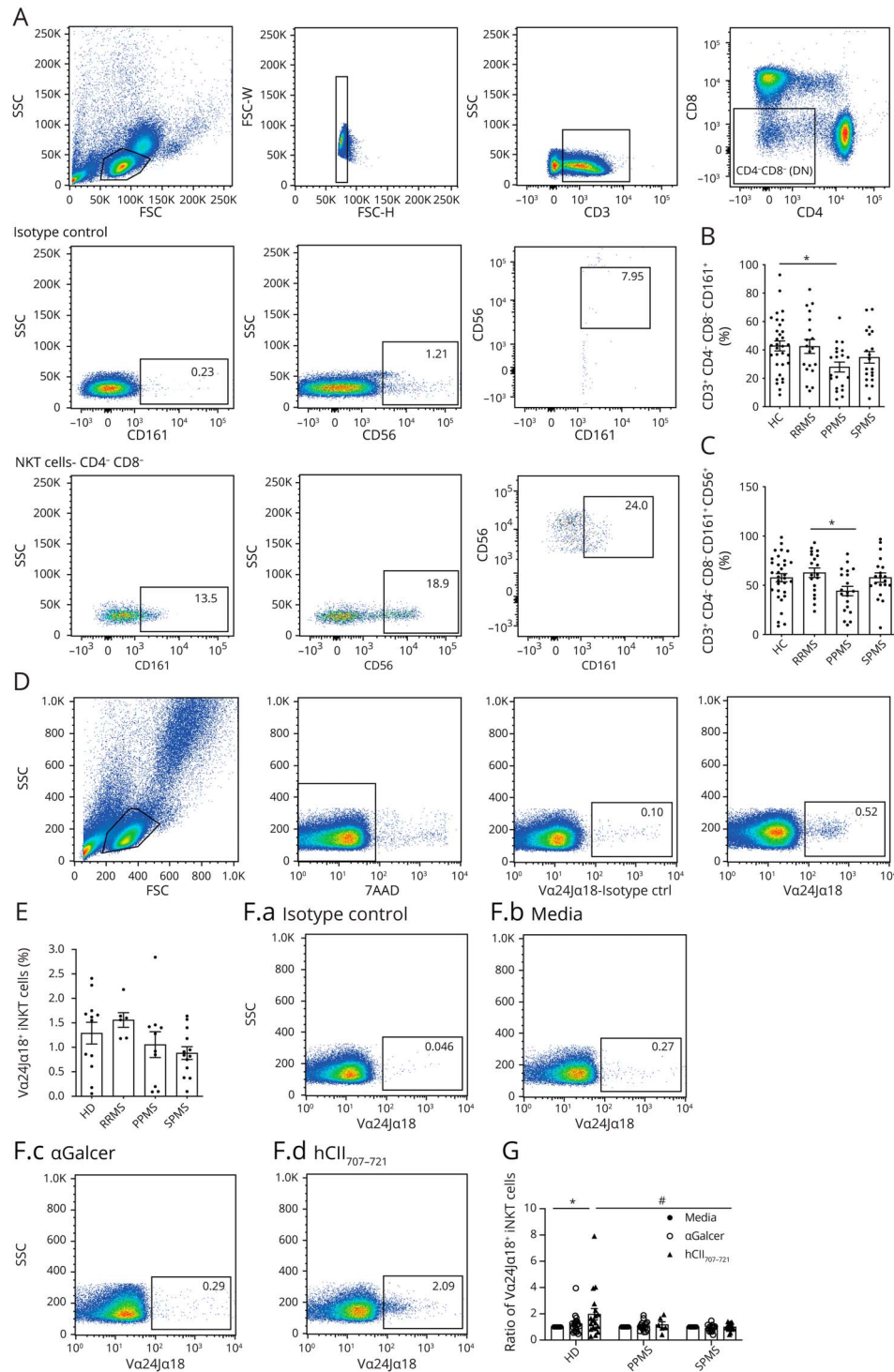
Next, we performed a Reactome pathway enrichment analysis of the upregulated genes to identify the molecular pathways that were associated with iNKT differentiation and function (Figure 2G). The resulting pathways reflected the cytotoxic nature of iNKT cells and included terms that are associated with important effector functions, such as degranulation (neutrophil degranulation), ROS production (ROS and RNA production in phagocytes), and interleukin signaling (interleukins 4, 13, and 10). Finally, we conducted gene set enrichment analysis (GSEA) of the iNKT profile vs convT, confirming the upregulation of iNKT effector functions using several pathway databases (Figure 2H).

Transcriptomic Profiling Reveals hCII707-721-Induced Activation and Proliferation of iNKTs

Compared with conventional nonactivated iNKT cells, hCII707-721-reactive iNKT cells upregulated 115 genes and downregulated 59 genes ($p < 0.05$, $|\log_{2}FC| > 0.5$) (Figure 3A–C, eFigure 2D, links.lww.com/NXI/A548, eTable 3, links.lww.com/NXI/A552), 33 of which were lncRNAs (Figure 3D, eTable 4, links.lww.com/NXI/A553)—the first indication that hCII707-721 activates human iNKT cells.

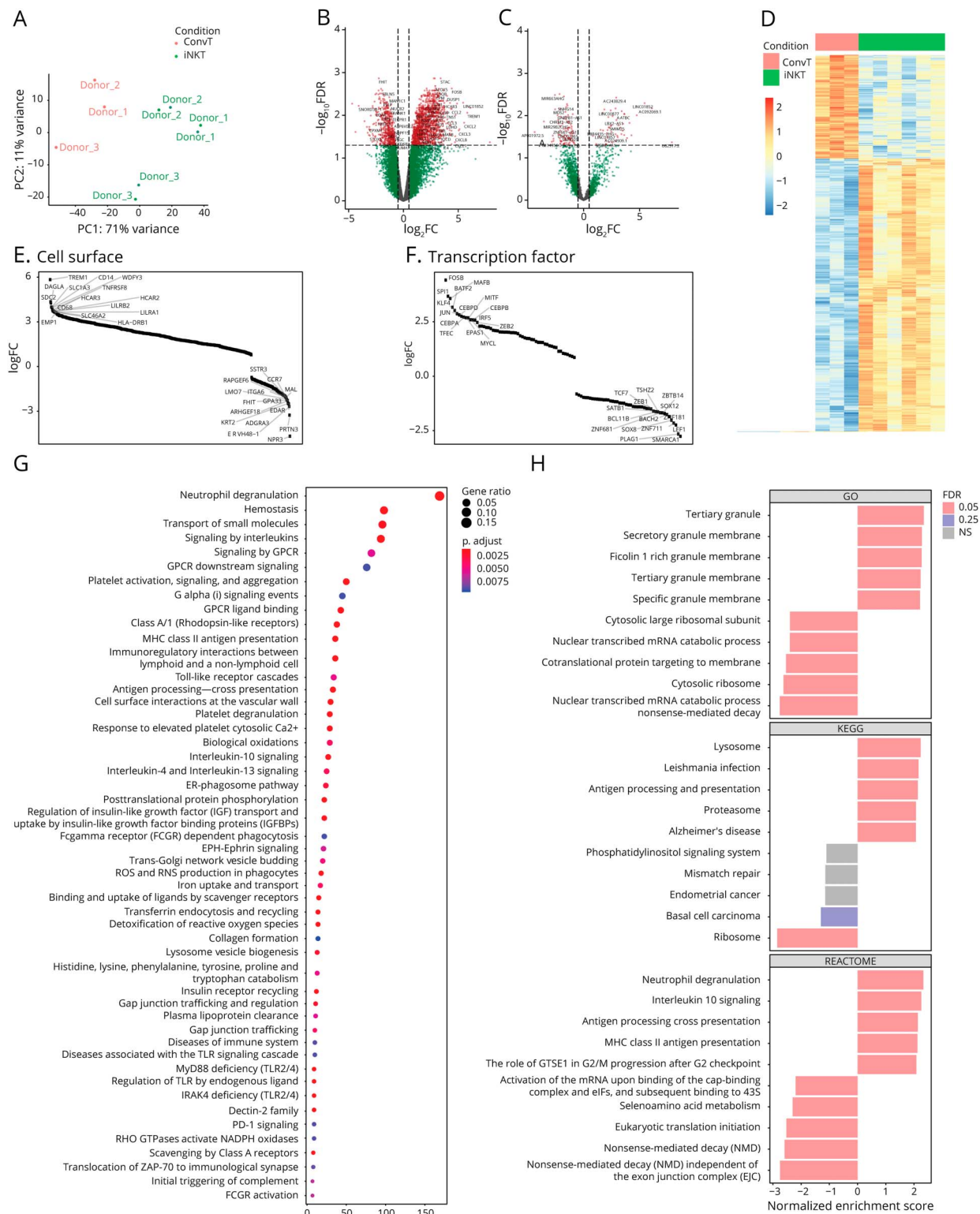
By GSEA, genes in pathways that were related to iNKT effector function were consistently upregulated. In 3 databases (GO, KEGG, and Reactome), proliferation-related pathways were the most highly upregulated (Figure 3E). Furthermore, the appearance of hematopoietic cell lineage by KEGG (FDR = 0.096) suggested that lineage commitment and differentiation occurred on activation (Figure 3E–F). In addition, hCII707-721 enhanced the effector function of iNKTs, based on their upregulation of TNF-related genes (Reactome-TNFs bind their physiologic receptors, FDR = 0.054) (Figure 3E).

Figure 1 NKT Cell Levels are Reduced and hClI₇₀₇₋₇₂₁-induced iNKT Cells Are Hyporesponsive in PPMS



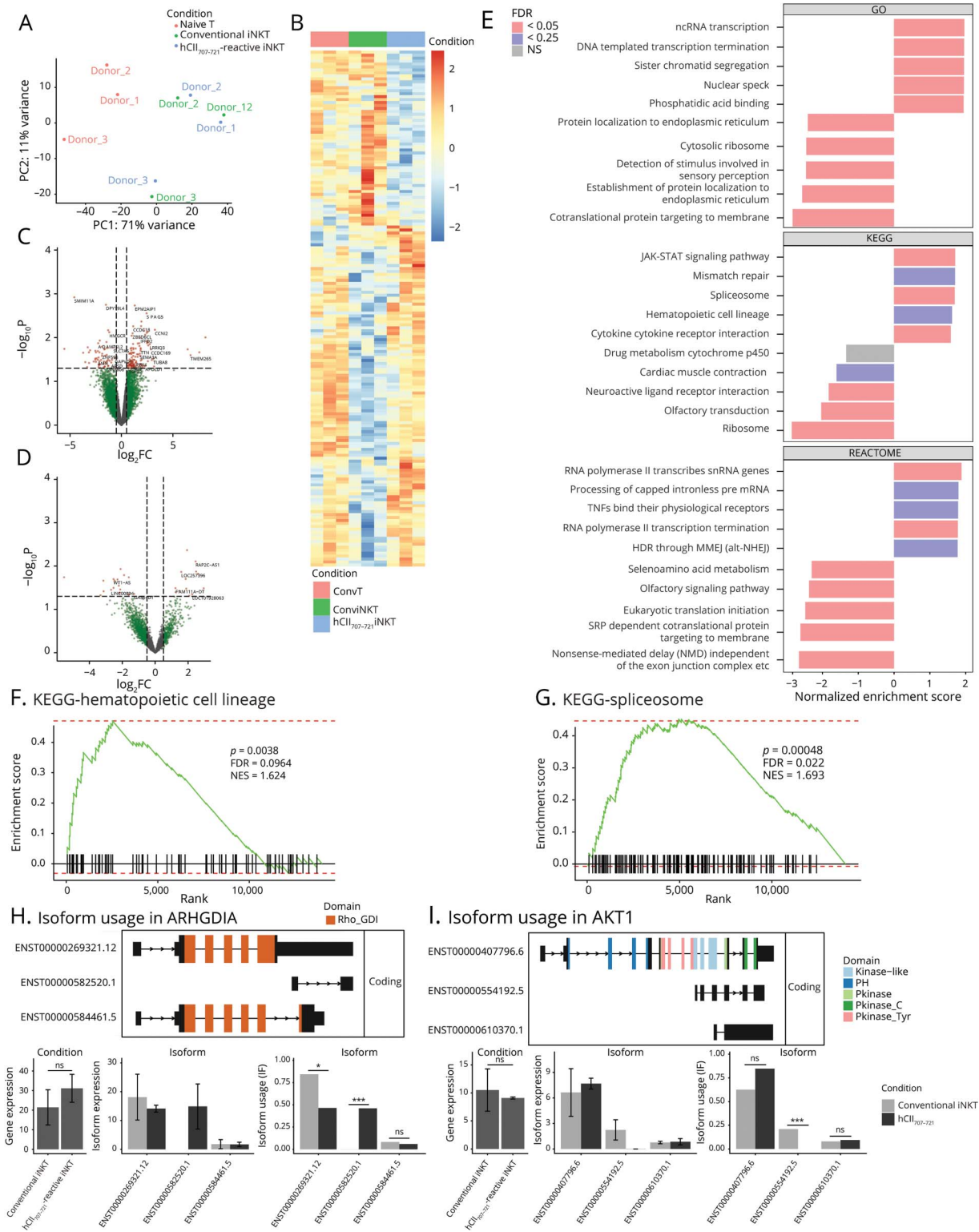
PBMCs were freshly isolated from each subject and stained with CD3, CD4, CD8, CD56, CD161, and Va24Ja18. (A) FACS gating strategy of CD3⁺CD4⁻CD8⁻ (DN) NKT cells and gating of DN CD56⁺CD161⁺ NKTs. (B) The percentage of DN NKT cells based on CD161 expression. (C) The percentage of DN NKT cells based on CD56 and CD161 expression. The bars indicate the mean ± SEM in HC (n = 32), patients with RRMS (n = 19), patients with PPMS (n = 20), and patients with SPMS (n = 20). (D) Gating of Va24Ja18⁺ iNKT cells from single and live lymphocytes compared with isotype control-stained cells. (E) The percentage of Va24Ja18⁺ iNKT cells in the various treatments. The bars indicate the mean ± SEM. HD (n = 12), RRMS (n = 6), PPMS (n = 10), and SPMS (n = 13). **p* < 0.05 One-way ANOVA with the Tukey multiple comparison test was applied. (F–G) PBMCs were cultured in vitro with α-GalCer (5 μg/mL), hClI₇₀₇₋₇₂₁ (100 μg/mL), or media for 48 hours. (F) Representative FACS dot plots showing gating of Va24Ja18⁺ iNKT cells in HD, PPMS, and SPMS (empty bar). Ratio is calculated as the percentage of treated Va24Ja18⁺ iNKT cells/percentage of Va24Ja18⁺ iNKT cells in media. The bars indicate the mean ± SEM, HD (n = 18), PPMS (n = 6–18), and SPMS (n = 15–18). Student *t* test was applied, **p* < 0.05 between control and hClI₇₀₇₋₇₂₁ in HDs. #*p* < 0.05 between HD and SPMS with hClI₇₀₇₋₇₂₁ treatment. ANOVA = analysis of variance; CD = cluster of differentiation; DN = double-negative; FACS = fluorescence-activated cell sorting; HC = healthy control; HD = healthy donor; NKT = natural killer T; PBMC = peripheral blood mononuclear cell; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SEM = standard error of mean; SPMS = secondary progressive MS.

Figure 2 iNKT Cells Have a Distinct Gene Expression Profile Compared With Conventional T Cells



Conventional T cells, conventional iNKT cells (without hCII707-721 activation), and hCII707-721-reactive iNKT cells (with hCII707-721 activation) purified from PBMCs were analyzed by RNA-seq. Conventional iNKT cells and hCII707-721-reactive iNKT cells were considered as a single group as iNKT cells in this analysis. $n = 3/\text{group}$. (A) Principal component analysis plot of convT (red) and iNKT cells (green). (B) Volcano plot of signature genes of iNKT cells vs conventional T cells (FDR < 0.05). (C) Volcano plot of differentially expressed lncRNAs between iNKT and conventional T cells (FDR < 0.05). (D) Heatmap of NKT signature genes. (E) A snake plot of cell surface markers of NKT cells highlighted the 15 upregulated and 15 downregulated genes. (F) A snake plot of transcription factor markers of NKT cells highlighted the 15 upregulated and 15 downregulated genes. (G) Top 50 enriched Reactome pathways among the genes that are upregulated in NKT cells vs convT. (H) GSEA showing the enriched pathways and molecular functions in iNKT cells per the GO, KEGG, and Reactome databases. convT = conventional CD4+ T cells; FDR = false discovery rate; GSEA = gene set enrichment analysis; iNKT = invariant NKT; NKT = natural killer T cell; PBMC = peripheral blood mononuclear cell.

Figure 3 hClI₇₀₇₋₇₂₁ Induces Proliferation and Activation in hClI₇₀₇₋₇₂₁-Reactive iNKT Cells



Treated iNKT cells were analyzed by RNA-seq compared with iNKT cells and convT. (A) Principal component analysis plot of conventional T cells, conventional iNKT cells, and hClI707-721-reactive iNKT cells. (B) Heatmap of differentially expressed genes in hClI707-721-reactive iNKT compared with iNKT. (C) Volcano plot showing the signature genes of hClI707-721-reactive iNKT and conventional iNKT ($p < 0.05$). (D) Volcano plot showing the lncRNAs differentially expressed between hClI707-721-reactive iNKT and conventional iNKT ($p < 0.05$). (E) GSEA of regulated GO, KEGG, and Reactome pathways in hClI707-721-reactive iNKT vs conventional iNKT cells. (F) Enrichment plot of KEGG-Hematopoietic cell lineage pathway in hClI707-721-reactive iNKT vs conventional iNKT cells ($p = 0.0038$, FDR = 0.0964, NES = 1.624). (G) Enrichment plot of KEGG-Spliceosome pathway in hClI707-721-reactive iNKT vs conventional iNKT cells ($p = 0.00048$, FDR = 0.022, NES = 1.693). (H) Differential isoform use of ARHGDI in hClI707-721-reactive iNKT cells. (I) Differential isoform use of AKT1 in hClI707-721-reactive iNKT cells. FDR = false discovery rate; GSEA = gene set enrichment analysis; iNKT = invariant NKT; NES = normalized enrichment scores.

Given the upregulation of the KEGG_SPLICEOSOME pathway (FDR = 0.022) (Figure 3G), we examined whether alternative splicing was induced by hCII707-721, finding 67 genes with significant differential isoform usage (eFigure 3A and B, links.lww.com/NXI/A549, eTable 5, links.lww.com/NXI/A554). Specifically, on stimulation, Rho GDP dissociation inhibitor alpha (ARHGDI1) changed to a non-functional isoform that lacked the functional domain, promoting Rho GTPase activation, which mediates T cell activation, ROS production, and cell migration^{25,26} (Figure 3H). Furthermore, hCII707-721 favored splicing of full-length AKT serine/threonine kinase 1 (AKT1), which mediates cell growth and proliferation²⁷ (Figure 3I).

Cytokine and Cytotoxic Molecule Profile of hCII707-721-Reactive iNKT Cells

Given that cytokine pathways were among the top KEGG pathways in hCII707-721-reactive iNKT cells (Figure 3D), we examined NKT cell signature cytokines in our RNA-seq data set. None of these cytokines was upregulated on stimulation with hCII707-721 (Figure 4A). The RNA-seq data were verified by intracellular cytokine measurements, merely showing significantly higher TNF α in hCII707-721-reactive iNKT cells vs conventional iNKT cells (gated on Va24J α 18⁺ cells) (Figure 4B–F). By ELISA, IFN- γ , IL-4, and IL-17F levels were minor (data not shown). Notably, unlike the reported massive induction of cytokines on administration of α -GalCer,²⁸ we found that hCII707-721 does not alter the expression or production of intracellular IFN- γ , TGF- β , or IL-17F (Figure 4B).

Perforin and granzyme B were expressed in nonactivated conventional iNKT cells (Figure 4G), consistent with previous reports.^{29,30} In addition, perforin expression did not differ between hCII707-721-reactive and nonactivated conventional iNKT cells (Figure 4G–I, M). Notably, granzyme B expression and secretion were reduced in hCII707-721-reactive iNKT cells vs conventional iNKT cells (Figure 4G, H, J, L, N). hCII707-721-reactive iNKT cells also produced significantly less granzyme B than α -GalCer-reactive iNKT cells (Figure 4L). FasL was upregulated on activation of iNKT cells with hCII707-721 compared with nonactivated iNKT cells, albeit insignificantly (Figure 4G–H, K, O).

hCII707-721-Reactive iNKT Cells Suppress Activated T Cells Through Fas/FasL and TNF α /TNFRI

As shown in Figure 5A–D, by in vitro suppression assay, the proliferation of activated responder T cells declined significantly in the presence of enriched autologous hCII707-721-reactive iNKT cells, without significantly affecting cell death (by 7AAD⁺ staining) (Figure 5E–F), such as murine CII707-721-reactive NKT cells,²¹ whereas nonactivated conventional iNKT cells effected no significant suppression (Figure 5G–K).

By transcriptomic analysis, hCII707-721-reactive iNKT cells upregulated Fas and TNF signaling (Figure 5L and M). Thus,

we hypothesized that hCII707-721-reactive iNKT cells suppress by engaging the Fas- FasL pathway. Suppression assay, using a blocking antibody to Fas on activated T cells, revealed the importance of this pathway (Figure 5N–O). Because hCII707-721-reactive iNKT cells have higher TNF levels and because cytotoxic lymphocytes induce cell death through TNF/TNFR,³¹ we also blocked TNFRI and TNFRII on activated T cells to determine the involvement of TNFRI in iNKT cell-mediated suppression (Figure 5N–O). Although the secretion of other cytokines by NKT cells is important for their function,^{3,6,7,32} we did not find any indication of their association with reactivity to hCII707-721, consistent with the absence of cytokine-mediated suppression in murine CII707-721-reactive NKT cells.²¹

Blocking Fas-FasL and TNFRI abolished the suppressive function of hCII707-721-reactive iNKT cells against activated T cell proliferation, whereas blocking TNFRII had no such effect (Figure 5N–O). However, the percentage of cell death (7AAD staining) was not affected (data not shown). These data suggested that both Fas-FasL and TNF–TNFRI signaling are involved in hCII707-721-reactive iNKT cell-mediated suppression.

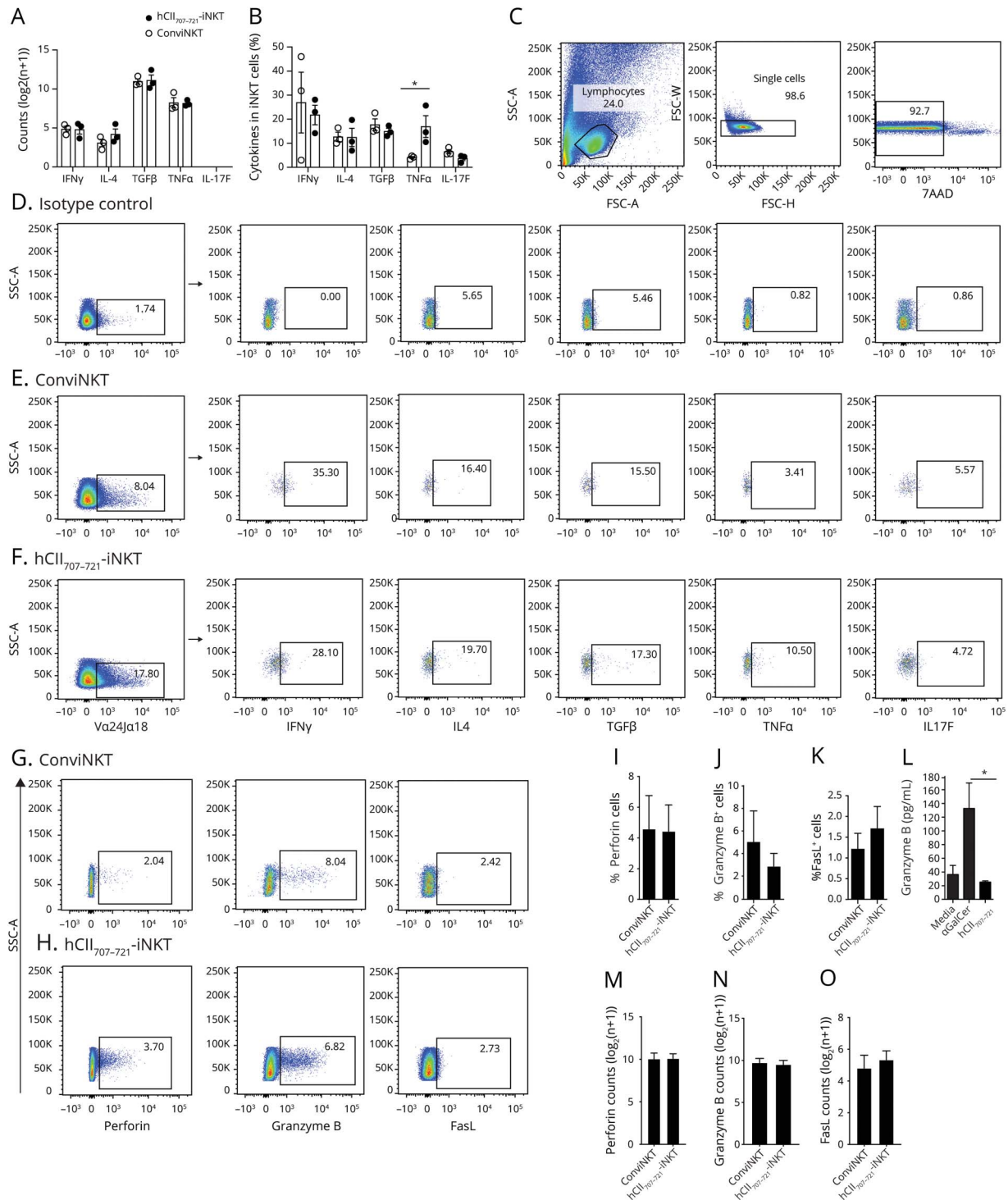
Discussion

The dysregulation of regulatory and suppressive immune cell types in autoimmune conditions³³ has spawned efforts to identify new such populations, culminating in the description of murine CII707-721-reactive NKT cells²¹ and circulating FoxA1⁺Treg cells in human blood.³⁴ In response to self-antigens and foreign antigens, NKT cells activate rapidly and develop proinflammatory and immunoregulatory functions, resulting in protective or harmful responses in many pathologies in mice and humans, including autoimmune disease, allergy, and cancer.^{6,10,33,35,36} This association underscores the importance of understanding the function and regulation of NKT cells to exploit them in the management of these diseases.³⁷

NKT cells have been implicated in MS, causing motor deficits, loss of coordination, cognitive and visual disturbances, and ultimately severe disability.¹¹ In keeping with their immunoregulatory functions, lower levels of and functional defects in NKT cell subsets have been reported in patients with MS.^{13,14} Conclusively, previous studies on this topic have been contradictory,^{13–15,22} likely because different markers were used to study NKT cell subsets in MS. Consistent with the evidence of a lower percentage of circulating NKT cells, in this study, we noted a lower percentage of various NKT cell populations in patients with PMS, including DN-NKT and iNKT cells. These findings indicate that NKT cells are protective in MS and that their absence is associated with disease progression.

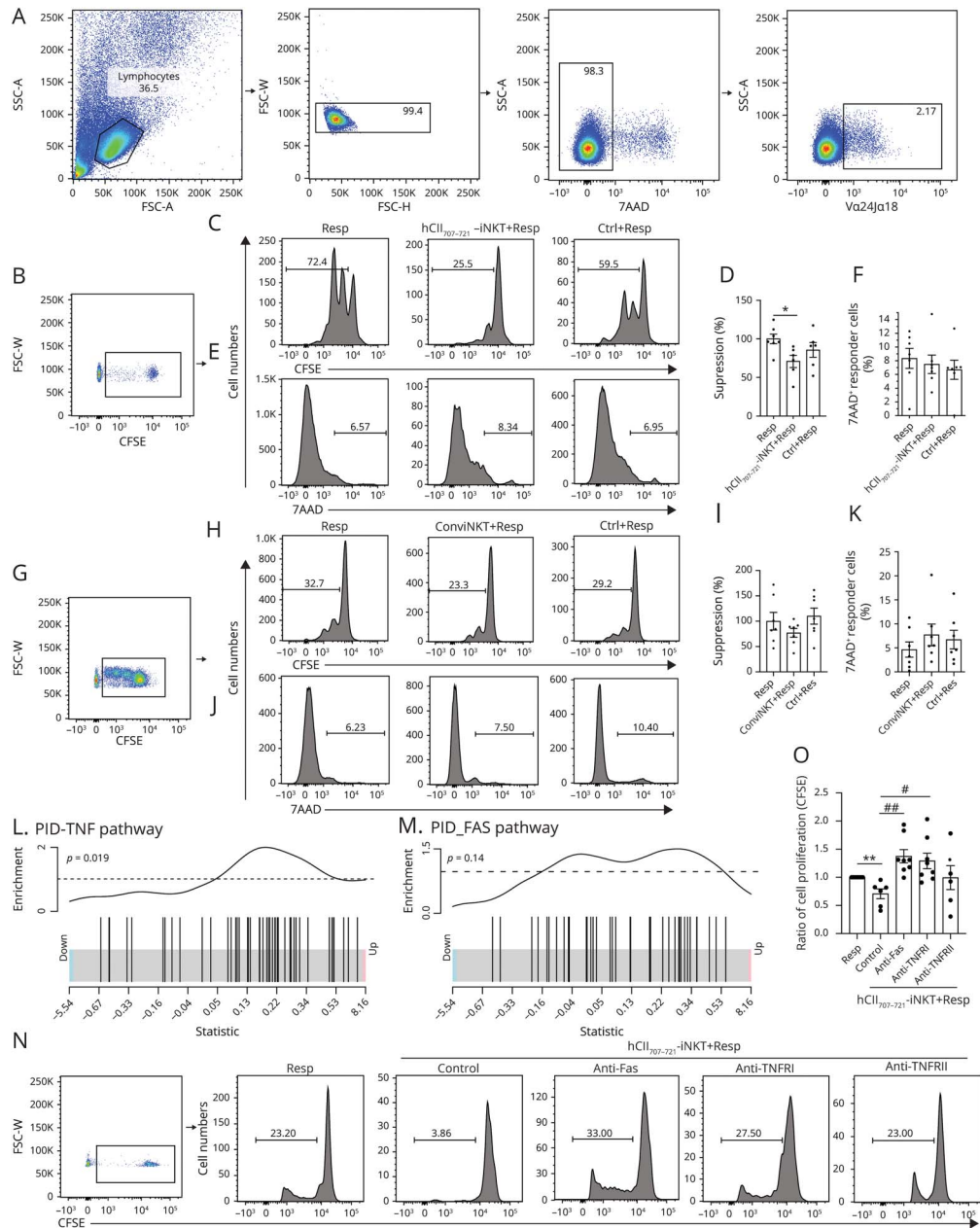
The major adverse effects of α -GalCer render it a suboptimal option for treating MS, perpetuating the unmet clinical need for therapies that activate protective NKT cells. Given the therapeutic potential of NKT cells and our description of

Figure 4 Cytokine and Cytotoxic Molecule Profiles of hCII₇₀₇₋₇₂₁-Reactive iNKT Cells Compared With Conventional iNKT Cells



PBMCs were isolated from HDs and treated with or without hCII707-721 for 48 hours. Cells were gated for Va24 α 18⁺ iNKT cells. (A) Normalized counts (from RNA-Seq) of IFN- γ ⁺, IL-4⁺, TGF β ⁺, TNF α ⁺, and IL-17F⁺ cells in iNKT cells. (B) The percentage of IFN- γ ⁺, IL-4⁺, TGF β ⁺, TNF α ⁺, and IL-17F⁺ cells in iNKT cells. Multiple *t* test, one per row was applied. **p* < 0.05 between hCII707-721 and untreated conventional iNKTs in HDs. (C) Dot plots showing the back-gating strategy for lymphocyte population, single cells, and live cells. (D) Dot plots of isotype controls for various cytokine stains. (E) Representative dot plots of cytokine stains from untreated conventional iNKT cells. (F) Representative dot plots of cytokine stains from hCII707-721-reactive iNKT cells. (G) Representative dot plots of cytokine stains from gated nontreated conventional iNKT cells. (H) hCII707-721-reactive iNKT cells. (I) The percentage of perforin⁺ (J) and granzyme B⁺ cells in gated iNKT cells. (K) FasL⁺ cells in gated iNKT cells. (L) Granzyme B production by -GalCer, hCII707-721, and untreated conventional iNKT cells after 48 hours of culture, (M) normalized counts (log₂-transformed normalized counts from DESeq2) of perforin expression by RNA-seq. (N) Normalized counts of granzyme B expression by RNA-seq. (O) Normalized counts of FasL by RNA-seq. The bars indicate the mean \pm SEM; *n* = 3–4. HD = healthy donor; IFN = interferon; IL = interleukin; NKT = natural killer T; PBMC = peripheral blood mononuclear cell; SEM = standard error of mean; TGF = transforming growth factor; TNF = tumor necrosis factor.

Figure 5 hCI1707-721-Reactive iNKT Cells Suppress Through Fas-FasL and TNF α -TNFRI



CFSE⁺ PBMC cells from HDs were activated with plate-bound anti-CD3 (10 μ g/mL) and soluble anti-CD28 (2 μ g/mL) for 48 hours as responders (Resp) and cocultured with purified autologous hCI1707-721-reactive Va24j α 18⁺ iNKT cells or control cells (Va24j α 18⁻ cells) for another 24 hours. (A) FACS gating strategy of back-gating and gating for Va24j α 18⁺ iNKT cells. (B) FACS plot of gating strategy for responder CFSE⁺ cells (hCI1707-721-reactive Va24j α 18⁺ iNKT cells as suppressors). (C) hCI1707-721-reactive Va24j α 18⁺ iNKT cells from PBMCs of a HD were enriched with magnetic beads before coculture with autologous activated responder T lymphocytes for functional suppression assay. Va24j α 18⁺ iNKT cells were obtained by treatment with hCI1707-721 (100 μ g/mL) for 48 hours (C-F) or from conventional Va24j α 18⁺ iNKT (G-K). (D) Representative histograms of CFSE⁺ gating in Resp CFSE⁺ responder T cells (Resp) cocultured with reactive hCI1707-721 Va24j α 18⁺ iNKT cells or control cells (Va24j α 18⁻ cells) for another 24 hours. (E) Representative histograms of 7AAD⁺ gating in Resp cocultured with reactive hCI1707-721 Va24j α 18⁺ iNKT cells or control cells (Va24j α 18⁻ cells). (F) The percentage of 7AAD⁺ dead cells in Resp CFSE⁺ responder T cells (Resp) were then cocultured with reactive hCI1707-721 Va24j α 18⁺ iNKT cells or control cells (Va24j α 18⁻ cells) for another 24 hours. (G) FACS plot of gating strategy for responder CFSE⁺ cells (conventional Va24j α 18⁺ iNKT cells as suppressors). (H) Conventional Va24j α 18⁺ iNKT cells cocultured with autologous activated responder T lymphocytes for functional suppression assay. Representative histograms of CFSE⁺ gating in Resp CFSE⁺ responder T cells (Resp) cocultured with conventional Va24j α 18⁺ iNKT cells or control cells (Va24j α 18⁻ cells) for another 24 hours, with percentages in (I). (J) Representative histograms of 7AAD⁺ gating in Resp cocultured with conventional Va24j α 18⁺ iNKT cells or control cells (Va24j α 18⁻ cells). (K) The percentage of 7AAD⁺ dead cells in Resp CFSE⁺ responder T cells (Resp) cocultured with conventional Va24j α 18⁺ iNKT cells or control cells (Va24j α 18⁻ cells) for another 24 hours. One-way ANOVA * p < 0.05. The bars indicate the mean \pm SEM, n = 7. (L) TNF pathway (PID, MSidDB) enrichment in hCI1707-721-reactive iNKT cells compared with conventional iNKT cells (FDR = 0.038). (M) FAS pathway (PID, MSidDB) enrichment in hCI1707-721-reactive iNKT vs conventional iNKT cells (FDR = 0.14). (N) Gating strategy for responder CFSE⁺ cells and proliferating CFSE⁺ cells. (O) The percentage of proliferating CFSE⁺ cells. Ratio of Resp-only (without neutralizing antibodies) set to 1; ratios calculated as percentage of proliferating CFSE⁺ cells in various cocultures/percentage of proliferating CFSE⁺ cells in Resp-only. The bars indicate the mean \pm SEM; n = 6-8. Unpaired student t test was applied; * p < 0.05 between Resp + iNKT (Control) and Resp-only. One-way ANOVA and Dunnett multiple-comparisons test: # p < 0.05, ## p < 0.01 between groups with blocking antibodies and control group. ANOVA = analysis of variance; CFSE = carboxyfluorescein succinimidyl ester; FACS = fluorescence-activated cell sorting; FDR = false discovery rate; HD = healthy donor; MS = multiple sclerosis; NKT = natural killer T; PBMC = peripheral blood mononuclear cell; PID = The Pathway Interaction Database; SEM = standard error of mean; TNF = tumor necrosis factor; TNFRI = TNF receptor I; 7AAD = 7-Aminoactinomycin D.

murine CII707-721-reactive NKT cells,²¹ we examined whether similar cells with possible clinical benefit existed in humans. This premise led to our identification of hCII707-721, revealing the existence of a novel iNKT cell population in healthy persons that is absent in patients with PMS and prompting us to characterize their molecular and suppressive properties. We have identified the first human self-peptide hCII707-721-reactive iNKT cell population in healthy humans and determined their transcriptional profile, which was borne out in their suppressive function.

Effector function pathways are significantly enriched in iNKT vs convT cells, including NK cell-mediated immunity, chemokine and cytokine responses, signal transduction, and cell motility and survival.³⁸ In our study, some such activities were validated in the general human iNKT cell signature, and others appeared only on activation of iNKT cells with hCII707-721.

Previous studies on the suppressive functions of iNKT cells have focused on the involvement of cytokines, based on the ability of NKT cells to robustly and rapidly produce cytokines that drive Th1, Th2, and Th17 cell differentiation and function.^{3,6,7,32} Although the “cytokine-cytokine receptors” interaction was among the major KEGG pathways that were induced by hCII707-721 in iNKT cells, the activation of iNKT cells by hCII707-721 was not associated with any specific cytokine pattern, except for higher TNF α levels. Consistent with this finding, our transcriptomic analysis of hCII707-721-reactive iNKT vs conviNKT cells implicated the TNF pathway as being significantly enriched. Although blocking TNFR1 on activated T lymphocytes did not hinder the antiproliferative effects of hCII707-721-reactive iNKT cells, TNF α -mediated TNFR1 signaling—a pathway that induces cell death³¹—contributed to their suppressive activity.

We found that the suppressive function of hCII707-721-reactive iNKT cells was also mediated by Fas-FasL signaling, as observed for murine CII707-721-reactive NKT cells.²¹ A similar mechanism was reported in the killing of tumor cells by GalCer-activated NKT cells,³⁹ consistent with the regulation of T cells by NKT cells through Fas/FasL.^{40,41} As our study has shown, cooperation between Fas/FasL and TNF α /TNFR1 is a potential suppressive mechanism of hCII707-721-reactive iNKT cells, as supported by the use of cytotoxic mechanisms by human NKT cells against a myelomonocytic leukemia cell line, including perforin/granzyme-B, TNF α , FAS-L, and TRAIL.⁴² Although 7AAD staining of cell death was not shown in the differences, it does not rule out a possibility that cell apoptosis could be involved in hCII707-721-reactive iNKT cell-mediated suppression.

Although NKT cells primarily recognize and react to glycolipids in the context of CD1 molecules, they can react to peptide antigens.⁴³ NKT cell regulation of immune homeostasis might depend on the recognition of self-antigens under stress, such as tissue damage, which causes the release of self-

antigens.³³ Notably, certain NKT cells react with sulfatide, a self-glycolipid in the myelin sheaths that coats and insulates nerve fibers in the CNS.⁴⁴ CD1-dependent activation by α -GalCer in mice induces all signature cytokines of various T helper subsets, demonstrating the cytokine storm that is elicited by this stimulation.⁴⁵ In contrast, the self-peptide hCII707-721 seems to be protective because of the consequent low cytokine output—except for TNF α , which initiates its TNFR1-mediated suppression—unlike with other CD1-restricted NKT cells, such as α -GalCer-reactive cells.

In summary, we found iNKT cells to be hyporesponsive to hCII707-721 in patients with PMS, reflecting a potential state of anergy in circulating self-reactive iNKT cells in these patients. Determining how anergic iNKT cells can be activated in patients with PMS might be helpful in designing hCII707-721-based therapies. Furthermore, direct clinical benefits have been obtained by stimulating or adoptively transferring NKT cells to correct defects in the NKT cell pool.^{8,9,17,21,46} Low production of IFN- γ , IL-4, and IL-17F by hCII707-721-reactive iNKT cells is a highly favorable attribute, avoiding, for example, shifts in polarizing T helper subsets, which are associated with pathology in MS.⁴⁷ Thus, based on our novel identification of endogenous self-antigen hCII707-721-reactive iNKT cells, approaches that use hCII707-721 to expand and activate hCII707-721-reactive iNKT cells have tremendous therapeutic potential to correct the defects of NKT cells in patients with MS and prevent disease progression.

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Disclosure

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Appendix (continued)

Name	Location	Contribution
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Jon Lundstrom, MSc	Biotech Research and Innovation Centre (BRIC), University of Copenhagen, Denmark	Major role in the acquisition of data; Analysis or interpretation of data
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