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Journal

The Journal of Immunology, 193(2)

ISSN

0022-1767

Authors

Lin, Lin
Ma, Chao
Wei, Bo
[et al.](#)

Publication Date

2014-07-15

DOI

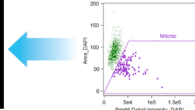
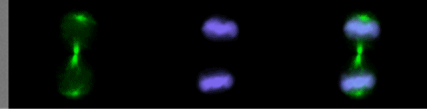
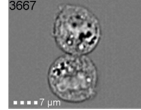
10.4049/jimmunol.1400093

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J Immunol 2014; 193:940-949; Prepublished online 16 June 2014;

doi: 10.4049/jimmunol.1400093

<http://www.jimmunol.org/content/193/2/940>

Supplementary Material <http://www.jimmunol.org/content/suppl/2014/06/16/jimmunol.1400093.DCSupplemental.html>

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Human NK Cells Licensed by Killer Ig Receptor Genes Have an Altered Cytokine Program That Modifies CD4⁺ T Cell Function

Lin Lin,^{*,†,1} Chao Ma,^{‡,1} Bo Wei,^{*} Najib Aziz,^{*} Raja Rajalingam,^{*} Susy Yusing,[§] Henry A. Erlich,[¶] Elizabeth A. Trachtenberg,^{||} Stephan R. Targan,[#] Dermot P. B. McGovern,^{#,**} James R. Heath,^{†,††,‡‡} and Jonathan Braun^{*,†}

NK cells are innate immune cells known for their cytolytic activities toward tumors and infections. They are capable of expressing diverse killer Ig-like receptors (KIRs), and KIRs are implicated in susceptibility to Crohn's disease (CD), a chronic intestinal inflammatory disease. However, the cellular mechanism of this genetic contribution is unknown. In this study, we show that the "licensing" of NK cells, determined by the presence of KIR2DL3 and homozygous HLA-C1 in host genome, results in their cytokine reprogramming, which permits them to promote CD4⁺ T cell activation and Th17 differentiation *ex vivo*. Microfluidic analysis of thousands of NK single cells and bulk secretions established that licensed NK cells are more polarized to proinflammatory cytokine production than unlicensed NK cells, including production of IFN- γ , TNF- α , CCL-5, and MIP-1 β . Cytokines produced by licensed NK augmented CD4⁺ T cell proliferation and IL-17A/IL-22 production. Ab blocking indicated a primary role for IFN- γ , TNF- α , and IL-6 in the augmented T cell–proliferative response. In conclusion, NK licensing mediated by KIR2DL2/3 and HLA-C1 elicits a novel NK cytokine program that activates and induces proinflammatory CD4⁺ T cells, thereby providing a potential biologic mechanism for KIR-associated susceptibility to CD and other chronic inflammatory diseases. *The Journal of Immunology*, 2014, 193: 940–949.

Natural killer cells are components of the innate immune system primarily known for cytolytic targeting of tumor cells and virally infected cells. Despite their apparent role in the development of many chronic inflammatory diseases (1–6), their functional role in pathogenesis is poorly understood. Recent human genome-wide association analysis has uncovered gene expression networks of NK cells implicated in inflammatory bowel disease (IBD) pathogenesis (7). However, due to the complex roles and functional proficiencies of NK cell subpopulations, the biologic mechanisms of their involvement in chronic inflammatory diseases, and the ones pertinent to IBD, remain uncertain (1–4, 8, 9).

Killer Ig-like receptor (KIR) genes are predominantly expressed by NK cells, and are one element of the receptor repertoire con-

trolling NK cell activation, proliferation, and effector functions that mediate surveillance and host defense for microbial infection and malignancy (10, 11). The genetic combination of KIRs with their respective HLA class I ligands is associated with multiple autoimmune disorders, infectious diseases, and cancers (5, 6). However, the functional mechanisms accounting for these disease associations, particularly for the inhibitory class of KIRs, are poorly understood. The KIR gene family is composed of 14 functional genes (seven activating, six inhibitory, one bifunctional) and 2 pseudogenes (12). The KIR locus exhibits haplotypes with extensive variations in number and types of KIR genes. Among the inhibitory KIRs, KIR2DL1 recognizes HLA-C2 allotypes, KIR2DL2 and KIR2DL3 recognize HLA-C1 allotypes, KIR3DL1 recognizes HLA-Bw4 (5, 12). Accordingly, functional pairings of cognate

^{*}Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095; [†]Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095; [‡]Division of Physics, Mathematics and Astronomy, California Institute of Technology, Pasadena, CA 91125; [§]Mattel Children's Hospital, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095; [¶]Children's Hospital Oakland Research Institute, Oakland, CA 94609; ^{||}Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305; [#]Translational Genomics Group, F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute, Los Angeles, CA 90048; ^{**}Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048; ^{††}NanoSystems Biology Cancer Center, California Institute of Technology, Pasadena, CA 91125; and ^{‡‡}Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

¹L.L. and C.M. contributed equally to this work.

Received for publication January 16, 2014. Accepted for publication May 15, 2014.

This work was supported by National Institutes of Health Grants PO1DK46763 (to S.R.T., D.P.B.M., and J.B.), CA119347 (to J.R.H.), 5 UO1 AI067068 (to H.A.E., E.A.T., and D.P.B.M.), DK062413 and DK046763-19 (to D.P.B.M.), and UL1TR000124 (to S.R.T., D.P.B.M., and J.B.) and the Cedars-Sinai F. Widjaja Inflammatory Bowel and Immunobiology Institute Research Fund. Project investigators were supported by the Helmsley Charitable Trust (to D.P.B.M.) and the European Union (to D.P.B.M.). This research was performed with material support provided by the Immune Tolerance

Network, an international clinical research consortium headquartered at the University of California, San Francisco, and supported by the National Institute of Allergy and Infectious Diseases and the Juvenile Diabetes Research Foundation.

L.L. coordinated and processed clinical samples, *ex vivo* culture, proliferation assay, and Th17 induction experiments; C.M. and L.L. designed the multiplex ELISA and SCBCs experiments and C.M. performed them; L.L. and C.M. performed statistical analysis for cytokine profile data; R.R. performed the HLA and KIR typing on healthy individuals and E.A.T. on Crohn's disease cohort; additional HLA and killer Ig receptor genotyping data for Crohn's disease patients was provided by H.A.E. and E.A.T., S.R.T., D.P.B.M., B.W., N.A., and S.Y. coordinated clinical sample collection; L.L. and J.B. conceived the study and directed the experimental design; and L.L., C.M., and J.B. wrote the manuscript. All authors reviewed and discussed the manuscript.

Address correspondence and reprint requests to Dr. Jonathan Braun, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, 925 Westwood Boulevard, Los Angeles, CA 90095-7209. E-mail address: JBraun@mednet.ucla.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: CD, Crohn's disease; IBD, inflammatory bowel disease; KIR, killer Ig-like receptor; PCA, principal component analysis; SCBC, single-cell barcode chip; UCLA, University of California, Los Angeles.

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HLA and KIR genes in an individual haplotype is quite heterogeneous, which confounds studies of their biologic function.

This study was prompted by the elevated genetic susceptibility for Crohn's disease (CD) in patients bearing the inhibitory KIR2DL2/3 with its cognate ligand HLA-C1 (13–15). This association was puzzling, because NK–target interaction via inhibitory KIR ligation suppresses NK effector function (2, 16). One potential explanation is the licensing effects of inhibitory KIRs during NK differentiation. Licensing is a maturational process, induced by signaling of select inhibitory KIR genes with their cognate HLA ligands during NK differentiation that confers expanded functional competence of NK cells. Because the presence of relevant pairs of inhibitory KIR and cognate HLA genes are stochastic, healthy individuals genetically vary in the presence or abundance of licensed NK cells. Also, although the known NK cell functions affected by licensing include augmented target cell killing and IFN- γ production (16, 17), there has been little study of NK licensing on other aspects of NK cell function: regulation of inflammation and adaptive immunity or the scope of licensing-related cytokine and chemokine production (18).

Mechanistic studies of human NK cell licensing are challenging due to the complex genetic composition of KIR–HLA combinations (12), and the conflicting roles inhibitory and activating KIRs play in licensing. Therefore, we focused on individuals homozygous for the KIR A haplotype (termed AA haplotype), a common genotype (~30% worldwide) which contains inhibitory KIRs for three key HLA class I ligands (*HLA-C1*, *HLA-C2*, and *HLA-Bw4*) but only one single activating KIR (12, 19). Different inhibitory KIR–HLA class I ligand pairs confer various levels of strength for NK licensing (Supplemental Table 1) (20). The most potent pair is KIR2DL3/*HLA-C1*; the second strongest pair is KIR3DL1/*HLA-Bw4*, whereas KIR2DL1/*HLA-C2* has minimal licensing effect. Because AA haplotype individuals have *KIR2DL3* and *KIR2DL1*, we consider individuals with homozygous *HLA-C1* (abbreviated as *HLA-C1C1*) strongly licensed individuals and individuals with *HLA-C1/HLA-C2* or homozygous *HLA-C2* (abbreviated as *HLA-C2⁺*) weakly licensed or unlicensed individuals.

Therefore, we aimed to investigate the potential of NK licensing in the KIR associated CD susceptibility. In this study, we show that NK cells from genetically licensed healthy subjects and CD patients efficiently augment antigenic CD4⁺ T cell proliferation, and this augmentation is mediated by soluble molecules secreted by licensed NK cells. Licensed NK cell supernatant also dramatically promotes Th17 cells, a signature CD4⁺ Th subset in CD. Multiplexed cytokine study of CD cohort demonstrated that genetically licensed and unlicensed NK cells exhibit consistent and distinct cytokine profiles, with licensed NK cells distinguished by high-output, proinflammatory, polycytokine expression. Selected cytokines among this output account for the capacity of licensed NK cells to efficiently augment antigenic CD4⁺ T cell proliferation and Th17 polarization.

Materials and Methods

Clinical samples

Clinical samples were collected according to protocols approved by the institutional review committee of Cedars-Sinai Medical Center (CSMC) and of University of California, Los Angeles (UCLA). CD patients, previously genotyped for HLA and KIR (13), were randomly chosen, consented and called back by CSMC. Of the 1306 patients, 455 are AA haplotype, 28 consented for callback blood donation, and all 20 subjects who returned for collection were included for study. Healthy donors were recruited at UCLA Clinical and Translational Research Laboratory and genotyped by the UCLA Immunogenetics Center.

Cell isolation

PBMCs were isolated by Ficoll–Paque (GE Healthcare, Chalfont St. Giles, U.K.) density gradient centrifugation. Human NK cells were purified either from whole blood using the RosetteSep Human NK cell enrichment

Cocktail or from PBMC using the Human NK cell negative selection kit (StemCell Technologies, Vancouver, BC, Canada). Human regulatory T cell-depleted T cells were purified from PBMC using a Human T cell enrichment kit and CD25-positive selection kit; CD4⁺ T cells were purified from PBMC using a Human T cell enrichment kit and CD4-positive selection kit (StemCell Technologies). The purity of isolated NK cells and T cells were confirmed to be above 90%.

NK–T cell coculture and blocking assays

Before coculture, round bottom 96-well plates were coated with anti-CD3/CD28 Ab (R&D Systems, Minneapolis, MN) in PBS at 1.5 $\mu\text{g ml}^{-1}$ at room temperature for 2 h or at 0.5 $\mu\text{g ml}^{-1}$ at 4°C overnight. T cells were stained with 0.2 μM CFSE (Invitrogen, Carlsbad, CA) and cocultured with NK cells for 3 d in 96-well plates at 1×10^6 cells ml^{-1} in presence of 2 ng ml^{-1} (26 IU) IL-2 with complete RPMI 1640 medium, containing 10% FBS, 100 IU ml^{-1} penicillin 100 $\mu\text{g ml}^{-1}$ streptomycin, 10 mM HEPES buffer, 2 mM glutamine (Cellgro, Manassas, VA), and 5×10^{-5} M 2-ME (Sigma-Aldrich, St. Louis, MO). Blocking Abs for OX40 ligand and 2B4 (CD 252 and CD244; R&D Systems) were added to the coculture at concentration of 10 $\mu\text{g ml}^{-1}$. Neutralizing Abs, for IL-6, IFN- γ , TNF- α , and isotype control mouse IgG1 κ (eBioscience, San Diego, CA), and their combinations were added to the coculture at 1.25 $\mu\text{g ml}^{-1}$. The recombinant cytokines IL-6, IFN- γ , and TNF- α (R&D Systems) was each added to a final concentration of 20 ng ml^{-1} , comparable to the concentration measured for these cytokines in NK cell 3-d culture media analyzed by multiplex ELISA chip. For Transwell assay, 24-well plates were used; NK cells were placed on the filter side of a 1.0- μm pore-sized Transwell (BD Falcon, San Jose, CA), and CFSE-stained T cells were placed on the plate side of the Transwell.

Th17 differentiation assay

Before culturing, round bottom 96-well plates were coated with 1 $\mu\text{g ml}^{-1}$ anti-CD3 (R&D Systems) in PBS for 2 h at room temperature, and washed with 5% human AB Serum RPMI 1640 medium (Lonza, Rockland, ME). Total CD4⁺ T cells were purified and stimulated with 0.2 $\mu\text{g ml}^{-1}$ soluble anti-CD28, primed with different percentages of NK supernatants, and in the presence or absence of various cytokine combinations. At day 6 or 7, CD4⁺ T cells were resuspended, washed once with media, and expanded with 2 ng ml^{-1} (26 IU) IL-2 plus the same conditions provided for priming. At day 14, the cells were stimulated with PMA/ionomycin and brefeldin A for 5 h. Cells were then surface stained with anti-CD3, intracellularly stained with anti-IFN- γ , anti-IL-22, and anti-IL-17A, followed by flow using LSRII (BD Biosciences, San Jose, CA).

Multiplex cytokine ELISA

CD NK cells were isolated using the Human NK cell negative selection kit (StemCell Technologies). NK cells were cultured for 3 d in round-bottom 96-well plate at 1×10^6 cells ml^{-1} in 2 ng ml^{-1} (26 IU) IL-2 with complete RPMI 1640 medium. Then media samples were collected, stored at –80°C. Before analysis, samples were thawed, concentrated four times, and assayed as one batch. The initial protein panel was chosen to incorporate immune function markers, consisting primarily of cytokines and chemokines that could be secreted by NK cells. The final NK secretion Ab panel was chosen to incorporate nonredundant secretions detectable at NK 3-d culture.

Single-cell multiplex cytokine analysis

Frozen aliquots of PBMC were thawed and recovered overnight. Bulk NK cells were purified using the Human NK cell enrichment kit; CD3[–]CD56^{dim} KIR2DL3⁺KIR3DL1[–]KIR2DL1[–] and CD3[–]CD56^{dim}KIR2DL3[–] NK subsets sorted by FACS. All cells were prepared on ice and immediately analyzed by a single-cell microchip. Briefly, the microchip proteomics platform is based on isolating individual or a small number of cells into several thousand ~600 pl volume microchambers, with each chamber equipped with a miniaturized Ab array. These chips permitted the simultaneous measurement of 19 protein markers in each microchamber. After loading onto the single-cell barcode chip (SCBC), the cells were stimulated with 5 ng ml^{-1} PMA and 500 ng ml^{-1} ionomycin for 12 h at 37°C, and the microchip was imaged to count cell numbers within each microchambers. After cells were washed off, the fluorescence readouts were generated by an ELISA immunoassay and were quantified using a GenePix 4400A array scanner and custom-built software algorithms.

Abs

The following Abs and cell tracer were used staining for flow analysis: FITC-conjugated anti-CD158b (BD Biosciences), anti-IFN- γ (eBioscience,

San Diego, CA); CFSE; PE-conjugated anti-IFN- γ (BD Biosciences), anti-TNF- α , anti-IL-22, anti-granzyme B (eBioscience), anti-GM-CSF (R&D Systems); PerCP-conjugated anti-CD3 (BD Biosciences); allophycocyanin-conjugated anti-CD158a (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD4 (BD Biosciences), anti-IL-17A (eBioscience); streptavidin-PerCP; PE-Cy7-conjugated anti-CD56, anti-CD14 (BD Biosciences), Vioblue-conjugated anti-3DL1 (Miltenyi Biotec, Bergisch Gladbach, Germany), eFluor 650NC-conjugated anti-CD3 (eBioscience); anti-mouse IgG κ /Negative Control Compensation Particles. The use of Ab for staining was performed per manufacturer's instructions with proper titrations. Abs used for cytokine assays are IL-2, IL-6, IL-10, IL-15, IL-13, CCL-4 (MIP-1 β), CCL-5, CXCL-10, CCL-2, CXCL-8, IFN- γ , TNF- α , TNF- β , granzyme B, TGF- β 1 (R&D Systems, Minneapolis, MN), IL-4, IL-12, GM-CSF, and perforin (eBioscience).

Flow cytometry and cell sorting

Phenotypic analysis of PBMC was performed using flow cytometry after staining of cells with fluorescence dye-conjugated Abs. Labeled cells were analyzed with a FACSCalibur flow cytometer using CellQuest software, or LSR II (BD Biosciences) using FACSDiva software (BD Biosciences) at UCLA Flow Cytometry Core, and data analysis was performed using FlowJo (Tree Star, Ashland, OR). Cells were sorted for CD3⁺CD56^{dim}KIR2DL3⁺KIR3DL1⁻KIR2DL1⁻ and CD3⁺CD56^{dim}KIR2DL3⁻, using Aria I equipped with FACSDiva software (BD Biosciences).

Statistical analysis and data access

Most data analyses involved comparison of continuous variables, so the Student two-tailed unpaired *t* test was used; *p* < 0.05 were regarded as significant. All cytokine data were normalized before biostatistical analysis. GraphPad Prism (San Diego, CA) was used for statistical analysis and graphing. To uncover and visualize patient groups based on these parameters, we used principal component analysis (PCA) and hierarchical clustering, box-plot and scatter-plot analysis were performed in R package using custom-written codes. Microchip data from this study is available from our laboratory Web site (<http://www.its.caltech.edu/~heathgrp/>).

Results

Licensed NK cells from HLA-C1C1 CD patients strongly promote the proliferation of autologous CD4⁺ T cells

Proinflammatory CD4⁺ Th cells are the main effectors in induction and perpetuation of intestinal inflammation (21, 22). As a major cellular component of innate immunity, NK cells demonstrably cross-talk with the adaptive immunity arm (3, 19, 23–25). Because NK cells can stimulate or inhibit T cell activation via multiple mechanisms (26–29), we first asked whether strongly and weakly licensed NK cells from CD patients differentially modulated T cell proliferation in vitro. We isolated blood NK cells and au-

Table I. CD patient demographics

UCLA_ID	HLA-Bw	HLA-C	Age (y)	Gender	Race	Age of Diagnosis (y)	Current Medication
CDKIR0804	Bw6	C1	63	F	Caucasian	19	None
CDKIR0805-88	Bw4, Bw6	C1	55	F	Caucasian	18	Methotrexate, darifenacin, ciprofloxacin
CDKIR0816	Bw4, Bw6	C1	50	F	Caucasian	26	Acetaminophen
CDKIR0822	Bw4, Bw6	C1	60	F	Caucasian	28	Adalimumab, clonazepam mesalamine, pravastatin, zolpidem, alprazolam
CDKIR0831	Bw6	C1	60	F	Caucasian	49	Glatiramer acetate, atorvastatin, bupropion, escitalopram, nitrofurantoin
CDKIR0919	Bw6	C1	49	F	Caucasian	12	Mesalamine, rifaximin, hydrochlorothiazide, alprazolam, acetaminophen, and hydrocodone
CDKIR0921	Bw4	C1	50	F	Caucasian	21	Methadone, fentanyl citrate, nexium
CDKIR1130	Bw6	C1	40	F	Caucasian	25	Escitalopram, lorazepam
CDKIR1207	Bw4	C1	44	M	Caucasian	30	6-Mercaptopurine, methotrexate, mesalamine
CDKIR012512-49	Bw4, Bw6	C1	55	F	Caucasian	33	6-Mercaptopurine, infliximab, citalopram
CDKIR0805-01	Bw6	C1, C2	48	F	Caucasian	26	Colesevelam, diphenoxylate and atropine, doxorubicin, cyclophosphamide, aprepitant, ondansetron, prochlorperazine
CDKIR0811	Bw4, Bw6	C1, C2	62	M	Caucasian	25	Infliximab
CDKIR0902	Bw4	C1, C2	41	F	Caucasian	33	Loestrin 4Fe, infliximab, propranolol, zolpidem
CDKIR0914	Bw4	C1, C2	63	F	Caucasian	31	Adalimumab, diphenoxylate and atropine, colestipol, simvastatin, levothyroxine, montelukast, belladonna alkaloids and phenobarbital, acetaminophen and hydrocodone, escitalopram, ibandronate, fioricet
CDKIR021712	Bw4, Bw6	C1, C2	40	M	Caucasian	22	Mesalamine, infliximab
CDKIR022412	Bw6	C1, C2	47	M	Caucasian	7	Lisinopril, fluoxetine, bupropion, prednisone
CDKIR0805-04	Bw4, Bw6	C2	31	M	Caucasian	22	6-Mercaptopurine
CDKIR0809	Bw4, Bw6	C2	46	M	Caucasian	30	Adalimumab, lisdexamfetamine, duloxetine, cyanocobalamin (b12)
CDKIR1014	Bw4	C2	42	M	Caucasian	17	6-Mercaptopurine, dicyclomine, zolpidem, alprazolam, amlodipine besylate, metoprolol, prednisone, dexlansoprazole, ondansetron, adalimumab
CDKIR012512-50	Bw4, Bw6	C2	43	M	Caucasian	33	Acetaminophen, cyclosporine, adalimumab, losartan potassium, cefuroxime

tologous T cells from CD patients (Table I), and cocultured them in the presence of immobilized anti-CD3/CD28 and IL-2 at $2 \text{ ng}\cdot\text{ml}^{-1}$ (26 IU). At day 3, CD4^+ T cell proliferation was measured via CFSE dilution (Fig. 1A). CD4^+ T cell proliferation was augmented linearly with the number of licensed NK cells present ($R^2 = 0.996$; Fig. 1B). Using linearity ($R^2 > 0.85$) as a quality control criterion, 12 patient assays were selected for genetic correlation analysis. At an NK:T ratio of 1:1, NK cells from *HLA-C1C1* patients were significantly more potent than those from *HLA-C2+* patients, and NK cells from *HLA-Bw4/Bw4* patients were significantly more potent than those from *HLA-Bw6+* individuals within the *HLA-C2+* subset. Thus, three distinct levels of NK function were observed: *HLA-C1C1 Bw6+* > *HLA-Bw4/Bw4* > *Bw6+/HLA-C2+* (Fig. 1C), and this order conformed to KIR licensing strength (Supplemental Table I) (20).

To investigate the nature of interaction between NK and CD4^+ T cells, we neutralized the surface costimulatory molecules 2B4 and OX40 ligand, expressed by NK cells to promote CD4^+ T cell activation (27–29). Surprisingly, augmentation was fully preserved when these surface molecules were blocked (Fig. 1D). To assess whether this interaction was contact-dependent at all, NK cells were separated from T cells using $1\text{-}\mu\text{m}$ pore Transwells, only allowing soluble mediators to communicate between the sides. Separating NK and CD4^+ T cells did not affect CD4^+ proliferation at all (Fig. 1E), suggesting that NK augmentation of CD4^+ T cell proliferation was mainly mediated by soluble molecules secreted by licensed NK cells.

NK cells from *HLA-C1C1* CD patients exhibit elevated proinflammatory cytokine production and polyfunctionality

Multiple cytokines and chemokines are produced by NK cells (18), but little is known about the scope of cytokine reprogramming by KIR-mediated NK licensing. Therefore, we cultured NK cells for 3 d under the same condition used for NK-T cell coculture experiments, and quantitated the level of a panel of cytokines in the NK supernatant using a multiplex ELISA chip, which can simultaneously analyze up to 19 cytokines (30, 31). When supernatants of NK cells from *HLA-C1C1* (strongly licensed) and *HLA-C2+* (weakly licensed) CD patients were compared, NK cells from *HLA-C1C1* patients were significantly more robust producers of nine cytokines (Fig. 2A). This was specific to NK cells, as cytokine production by T cells was indistinguishable between *HLA-C1C1* and *HLA-C2+* patients (data not shown). The core differences resided in CCL-5 and MIP-1 β (chemokines important for neutrophil and T cell recruitment); and, IFN- γ , TNF- α , IL-6, and IL-4 (proinflammatory cytokines known to play a role in CD) (Fig. 2A). In contrast, both types of NK cells produced negligible IL-12, IL-15, or IL-10 (Fig. 2A), because their levels were at or below the background detection threshold. Hierarchical clustering (Fig. 2B) showed that *HLA-C1C1* and *HLA-C2+* patients were completely separated, demonstrating their distinct secretion capacities. To assess native NK cell activation state (CD69 expression), we compared six subjects (three *HLA-C1C1* and three *HLA-C2+*). At the time of isolation, the frequency of CD69^+ cells was significantly elevated in *HLA-C1C1* NK cells compared with *HLA-C2+* NK cells (data not shown, $p = 0.018$); CD69 expression was in most cultures stable after 24 h in low-dose IL-2. This observation suggested a potential positive correlation between CD69 expression and licensing-induced NK cell cytokine capacity.

NK cells from *HLA-C1C1* healthy subjects have comparable CD4^+ T cell proliferation-augmenting capability

To investigate whether NK cells from AA haplotype licensed healthy donors have similar levels of functionality as licensed CD

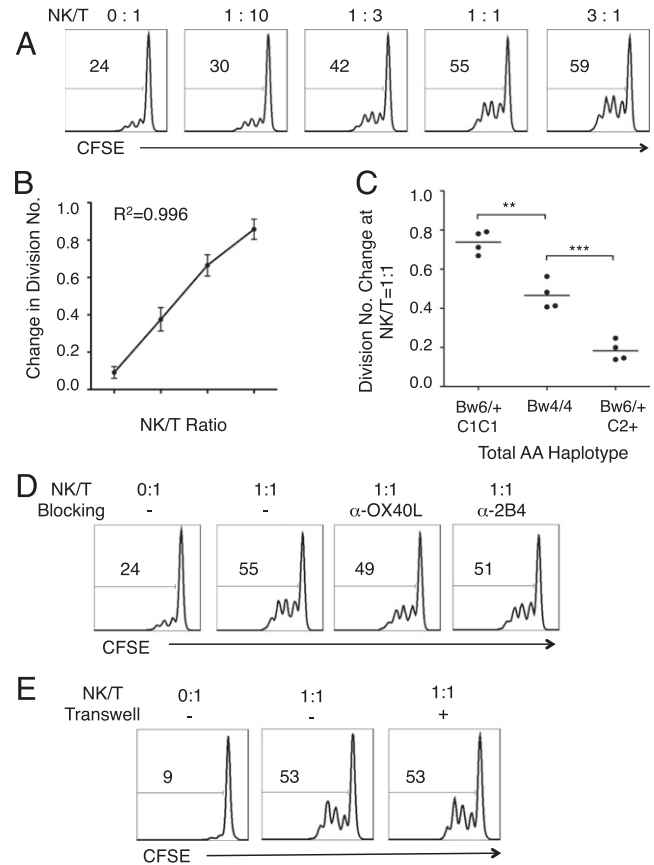


FIGURE 1. NK cells from genetically licensed CD patients strongly augment autologous CD4^+ T cell proliferation. NK cells and autologous T cells were isolated from AA haplotype CD patient peripheral blood, stimulated with anti-CD3 and anti-CD28, and cocultured in 2 ng ml^{-1} (26 IU) IL-2 for 3 d. (A) Histograms of CD4^+ T cell CFSE dilution after coculturing with NK cells at the NK/T ratios as indicated, for a representative C1C1 CD patient (gated on $\text{CD4}^+\text{CFSE}^+$ cells). The number within each graph indicates the percentage of cells proliferated. (B) Correlation between NK/T ratio and change in CD4^+ T cell division number in log scale, calculated as mean CFSE intensity at coculture/mean CFSE intensity of T cell alone. (C) Comparison of change in CD4^+ T cells division number at NK/T = 1:1, among C1C1Bw6 $^+$, Bw4/Bw4, and C2 $^+$ Bw6 $^+$ AA haplotype patients ($n = 4$, Student t test, two-tailed. $**p < 0.005$, $***p < 0.0005$). (D) Histograms of CD4^+ T cell CFSE dilution in the absence of (left two) or in the presence (right two panels) of the indicated blocking Abs at $10 \mu\text{g ml}^{-1}$ (gated on $\text{CD4}^+\text{CFSE}^+$ cells). (E) Histograms of CD4^+ T cell CFSE dilution at the indicated NK/T ratio without physical separation of NK cells and T cells (left two panels) or with separation by $1.0\text{-}\mu\text{m}$ -pore size Transwells (right panel) (gated on $\text{CD4}^+\text{CFSE}^+$ cells). The numbers in each histogram indicate the percentage of proliferating cells.

patients, we assessed their effects in CD4^+ T cell coculture. Using the same coculture assays described earlier, we observed that CD4^+ T cell proliferation increased linearly with the number of licensed NK cells present in the coculture ($R^2 = 0.949$; Fig. 3A, 3B). At an NK:T ratio of 1:1, the effect on CD4^+ T cells by NK cells from the 2 *HLA-C1C1* healthy subjects was comparable that that of NK cells from *HLA-C1C1* patients (Figs. 3C, and 1C).

NK cells from *HLA-C1C1* patients contain a subset dominating the response

We speculated whether this secretion difference reflected a homogeneous functional change in the NK cell population, or instead a mosaic of cellular heterogeneity. Therefore, we assessed cytokine secretion profiles of individual NK cells at the single cell level

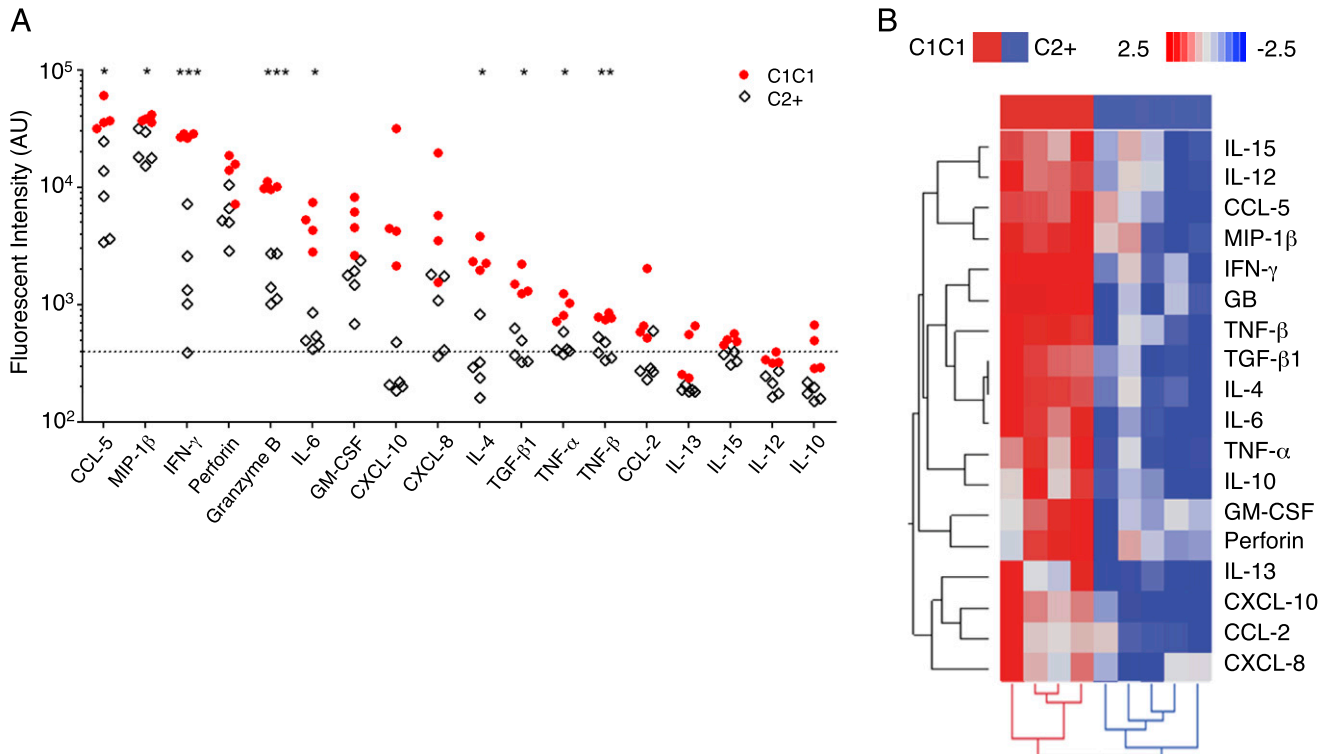


FIGURE 2. NK cells from *HLA-C1C1* patients have distinct cytokine secretion patterns compared with those from *HLA-C2+* patients in bulk culture. **(A)** Univariate comparison of cytokine production level of bulk culture NK cells from *HLA-C1C1* CD patients with (licensing, solid dot) and *HLA-C2+* patients (unlicensed, open square) genotypes. The vertical axis shows the fluorescence intensity. ($n = 4-5$, p values are calculated using two-tailed Student t test, adjusted for multiple comparison by false discovery rate, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$). The dash line indicates the detection threshold. Secretion profiles were measured by multiplex ELISA. **(B)** Hierarchical clustering of the bulk cytokine production profile of NK cells from *HLA-C1C1* (red) and *HLA-C2+* (blue) CD patients. Each row represents one protein indicated on the right, and each column represents one patient. ($n = 4-5$).

using SCBCs (30), a high-throughput microfluidics platform. With SCBCs, single cells or a small number of cells are separated into thousands of microchambers on a chip, where the production of 19 cytokines is simultaneously and independently measured during

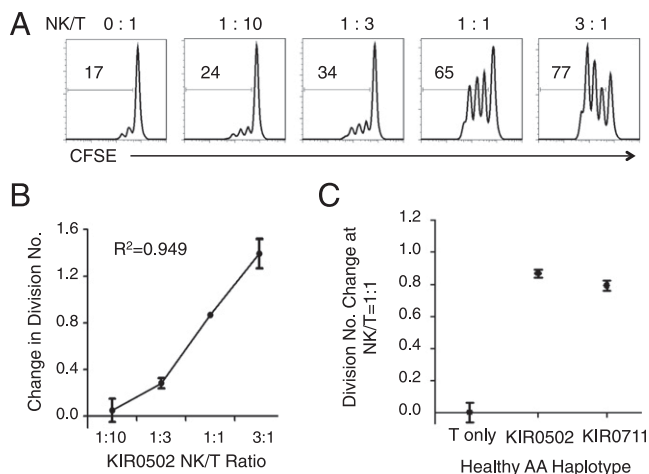


FIGURE 3. NK cells from *HLA-C1C1* healthy subjects have comparable $CD4^+$ T cell-augmenting capacity as *HLA-C1C1* CD patients. **(A)** Histograms of $CD4^+$ T cell CFSE dilution after coculturing with NK cells at the NK/T ratios as indicated, for a representative *HLA-C1C1* healthy subject (gated on $CD4^+CFSE^+$ cells). The number within each graph indicates the percentage of cells proliferated. **(B)** Correlation between NK/T ratio and change in $CD4^+$ T cell division number in log scale, calculated as mean CFSE intensity at coculture/mean CFSE intensity of T cell alone. **(C)** Change in $CD4^+$ T cells division number at NK/T = 1:1 from two *HLA-C1C1* healthy subjects.

a 12-h period. This technology has been extensively validated, and its utility in studying immune cell response has been demonstrated, as well as compared with standard flow assays (30).

We compared single NK cell cytokine secretion between two *HLA-C1C1* and two *HLA-C2+* CD patients, NK cells from *HLA-C1C1* patients exhibited a higher output of multiple cytokines, including TNF- α , MIP-1 β , GM-CSF, IFN- γ , IL-2, IL-6, and CXCL-10 (Fig. 4A, 4B, Supplemental Fig. 1). PCA of the single-cell data showed that NK cells with from *HLA-C1C1* patients contained a subset that produced effector proteins (CCL-5, TNF- α , IFN- γ , MIP-1 β , and IL-6), which in contrast was barely detected in NK cells from *HLA-C2+* patients (Fig. 4C). When the composition of cells producing one, two, three, four, five, and more than five cytokines was characterized, we observed a more polyfunctional phenotype of NK cells from *HLA-C1C1* patients (Fig. 4D). This analysis established that NK cells from *HLA-C1C1* patients exhibited a higher output of multiple cytokines and greater cytokine polyfunctionality at the single-cell level, distinguished by a subset producing effector proteins (CCL-5, TNF- α , IFN- γ , MIP-1 β , and IL-6). These findings together demonstrated that NK cells from CD patients with licensing genotypes were reprogrammed in a mosaic fashion for enhanced production of cytokines contributing to a chronic inflammatory state in vivo.

NK cells expressing KIR2DL3 are predominately responsible for the elevated cytokine production and polyfunctionality in HLA-C1C1 individuals

A simple mechanistic explanation for the mosaic pattern of NK cytokine expression is the underlying developmental heterogeneity of NK cells. During NK cell development, KIRs are stochastically expressed, resulting in a composite of licensed and unlicensed NK

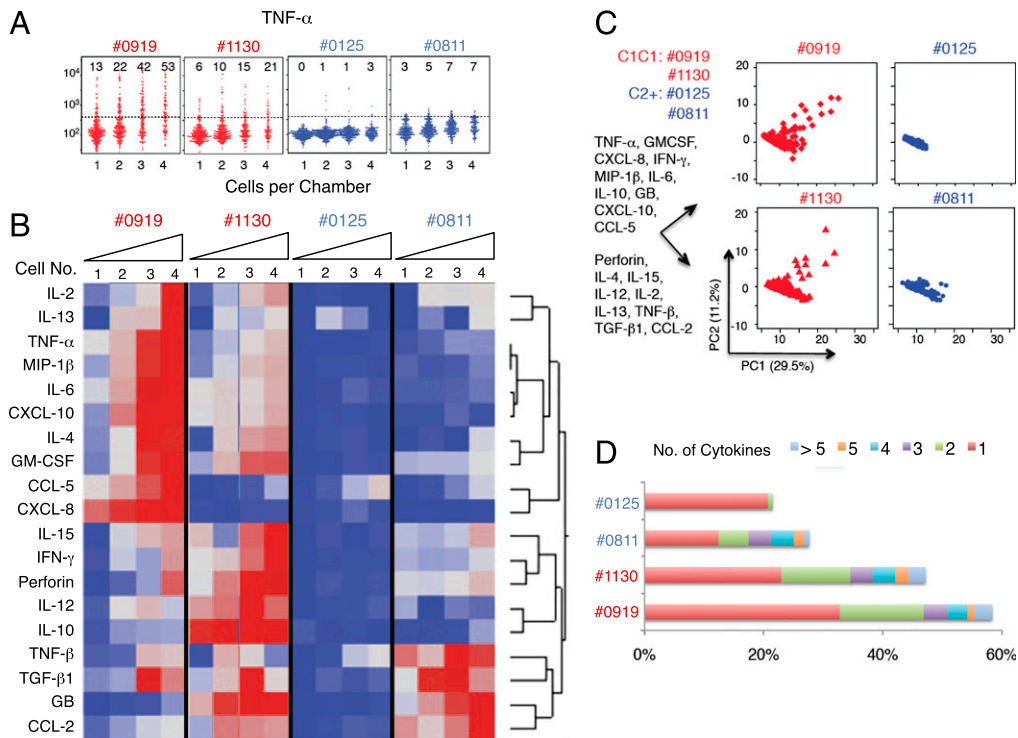


FIGURE 4. NK cells from *HLA-C1C1* patients contain a subset of NK cells polarized for proinflammatory cytokines production. **(A)** Univariate comparison of TNF- α production of NK cells from licensed (numbers 0919 and 1130, red) and unlicensed (numbers 0125 and 0811, blue) CD patients. The numbers in each graph indicate the percentage of microchambers that are positive for TNF- α signals. **(B)** Heat map of cytokine secretion capacity for all the cytokines and all the CD patients analyzed. Each row represents one cytokine, and each column represents the percentage of microchambers that are positive for the cytokine. The color scale shows the difference in SD. **(C)** PCA single NK cell measurements from the four CD patients. The percentage of variation explained by each component is shown in parentheses for each axis. The composition for each component is indicated on the left of the plots. **(D)** Bar graph of NK cell polyfunctionality. Different colors denote the percentages of single NK cells producing one, two, three, four, five, or more than five cytokines.

cells in individuals with licensing genotypes (32). Accordingly, when such a subject is *HLA-C1C1*, *KIR2DL3*⁺ NK cells are licensed, and the *KIR2DL3*⁻ NK cells are unlicensed. We therefore sorted licensed (*CD3*⁻*CD56*^{dim}*KIR2DL3*⁺*KIR3DL1*⁻*KIR2DL1*⁻) and unlicensed (*CD3*⁻*CD56*^{dim}*KIR2DL3*⁻) subsets (>95% purity) and evaluated their cytokine production at the single-cell level using SCBCs. Compared with *KIR2DL3*⁻ NK cells, *KIR2DL3*⁺*KIR3DL1*⁻*KIR2DL1*⁻ NK cells coexpressed elevated levels of CCL-5, MIP-1 β , IFN- γ , and TNF- α (Fig. 5A). To look at the data from a different angle, the majority (65%) of *KIR2DL3*⁻ NK cells did not secrete any cytokine. In contrast, 99% of *KIR2DL3*⁺*KIR3DL1*⁻*KIR2DL1*⁻ NK cells secreted at least one cytokine, with one-third expressing two cytokines (typically CCL-5⁺MIP-1 β ⁺), another third expressing three cytokines (mostly IFN- γ ⁺MIP-1 β ⁺CCL-5⁺); a substantial fraction (10%) produced four cytokines (Fig. 5B).

Analysis of ~1500 single NK cells resolved them into two clusters (hierarchical clustering in Fig. 5C and PCA in Supplemental Fig. 2). One cluster, predominated by *KIR2DL3*⁺*KIR3DL1*⁻*KIR2DL1*⁻ NK cells was polarized toward a proinflammatory state (effector proteins such as TNF- α , IFN- γ , and chemokines). A second cluster, predominated by *KIR2DL3*⁻ NK cells, was polarized toward a more regulatory state (including IL-4, TNF- β , TGF- β 1, and IL-10). Taken together, licensing mediated by *KIR2DL3/HLA-C1* interaction conferred proinflammatory immune mediator production program in NK cells.

IFN- γ , TNF- α , and IL-6 account for the capacity of licensed NK cells to augment CD4⁺ T cell proliferation

To evaluate whether the cytokines produced by licensed NK cells could indeed promote CD4⁺ T cell proliferation, we neutralized

IL-6, IFN- γ , TNF- α , or their combinations in NK-T cocultures (Fig. 6). Neutralization of TNF- α alone had a great impact on CD4⁺ T cell proliferation, and this effect was specific compared with IgG κ 1 isotype control. Neutralization of IL-6 or IFN- γ alone had measurable but modest effects, but their combination markedly reduced CD4⁺ T cell proliferation, suggesting synergistic interaction between them. We further evaluated T cell proliferation in the absence of NK cells, in which exogenous cytokines were added at 20 ng·ml⁻¹, comparable to that produced by licensed NK cells (data not shown). IFN- γ or TNF- α had marginal effects, but the addition of IL-6 or all three greatly facilitated CD4⁺ T cell proliferation. This indicates that IL-6 might not be necessary to augment CD4⁺ T cell proliferation in the presence of other cytokines produced by licensed NK cells, but it was sufficient to carry the proliferating effect alone. These cytokine depletion and addition results demonstrated that CD4⁺ T cell proliferation mediated by NK cells does not rely solely on one particular cytokine but rather depends on the balance of multiple key cytokines.

Secreted products of NK cells from HLA-C1C1 individuals potentially promoted Th17 differentiation

Th17 cells are crucial drivers for multiple chronic inflammatory diseases, including CD (33, 34), but there is little information about if or how NK cells might affect Th17 induction or activity. The foregoing results indicated that NK cells from *HLA-C1C1* individuals are robust producers of several cytokines, notably IL-6, which is critical for Th17 differentiation (33, 35, 36). After validating conditions for cytokine induced formation of IL-17A⁻ and IL-22⁻producing Th17 cells (Supplemental Fig. 3A), we determined whether the supernatant of NK cells from *HLA-C1C1* healthy individuals could promote Th17 differentiation from total

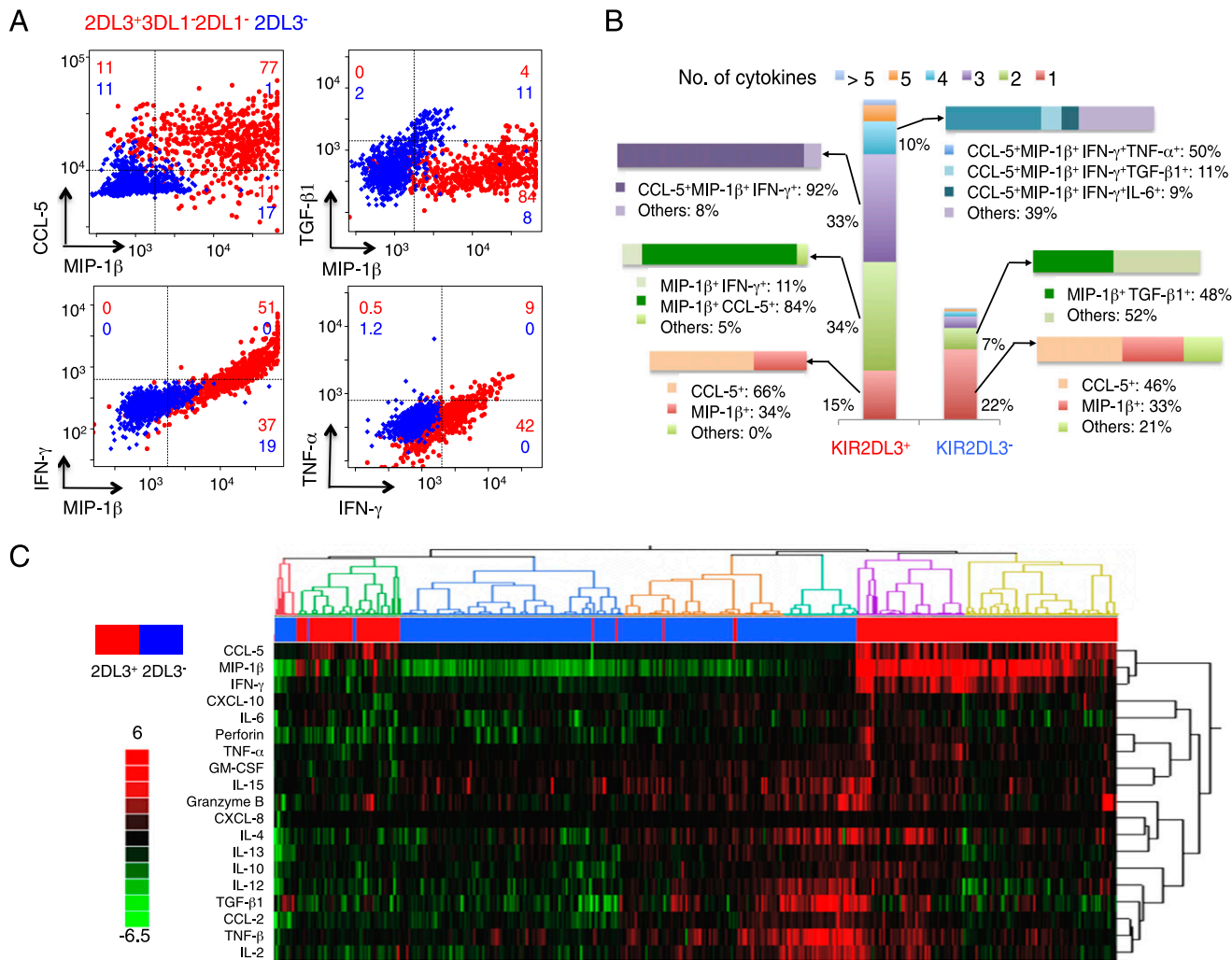


FIGURE 5. KIR2DL3⁺ NK cell subset is responsible for the enhanced proinflammatory cytokine production seen in *HLA-C1C1* individuals. Peripheral blood NK cells from an AA haplotype *HLA-C1C1* healthy donor were sorted for KIR2DL3⁺KIR3DL1⁻KIR2DL1⁻ (licensed) and KIR2DL3⁻ (unlicensed) NK subsets, stimulated with PMA/ionomycin, and analyzed using SCBC. **(A)** Two-dimensional scatter plot of representative cytokine production levels from licensed (red) and unlicensed (blue) NK cell subsets. Axis units are fluorescence intensity, and the value in each gated quadrant is the cell percentage. **(B)** Polyfunctionality plot showing the composition of NK subsets secreting zero, one, two, three, four, five, and more than five cytokines in licensed (red) and unlicensed (blue) NK cell subsets. The frequency for each major category is shown. **(C)** Hierarchical clustering of cytokine measurements from single licensed (red) and unlicensed (blue) NK cell subsets. Each column represents one single cell, and each row presents one cytokine. The color scale shows the difference in SD. Results are representative of three independent experiments.

CD4⁺ T cells. Indeed, NK supernatants from an *HLA-C1C1* healthy individual (AA haplotype), titrated into CD4⁺ T cell cultures, strongly induced the levels of IL-17A⁺, IL-22⁺, and IL-17A⁺IL-22⁺ T cells (Fig. 7A, 7B). These effects were observed with licensed NK supernatants alone, or in combination with IL-23, IL-1β, or both IL-1β and IL-23 (Supplemental Fig. 3B).

Using the IL-23 plus NK supernatant condition, we analyzed NK cell supernatants from three different healthy subjects with a licensing genotype (AA haplotype, *HLA-C1C1*). All of them showed strong Th17 differentiation responses (Fig. 7C). Among the licensed NK-derived cytokines, IL-6 is a likely candidate to promote Th17 cell differentiation. However, blocking IL-6 alone didn't detectably affect Th17 differentiation (Supplemental Fig. 3C). These findings demonstrate the capacity of licensed NK cells to secrete immune mediators that can strongly promote Th17 differentiation, either alone or synergistically with IL-23 and IL-1β.

Discussion

Through a combination of advances in genotyping technologies, statistical advances and collaborative efforts, there have been

spectacular advances in the understanding of the genetic contribution to complex diseases. Arguably, studies into the IBDs, CD, and ulcerative colitis have yielded the most successful results with >160 susceptibility loci now identified through a succession of genome-wide and more targeted approaches (34, 37, 38). The rapidity of gene discovery in IBD has far outpaced an understanding of both the functional and clinical consequences of associated variants and bridging this “genes to biology” gap requires significant investment and progress if the full benefits of genetic advances are to be realized. We and others have previously identified associations between IBD and genetic variation at the KIR locus (7, 13, 14). KIR genes are predominantly expressed by NK cells and are one element of the receptor repertoire controlling NK cell activation, proliferation, and effector functions that mediate surveillance and host defense for microbial infection and malignancy (10, 11). The relevance of this locus together with the role of NK cells in the development of IBD has recently been further highlighted with the publication of the IBD genome-wide association studies/ImmunoChip study (7).

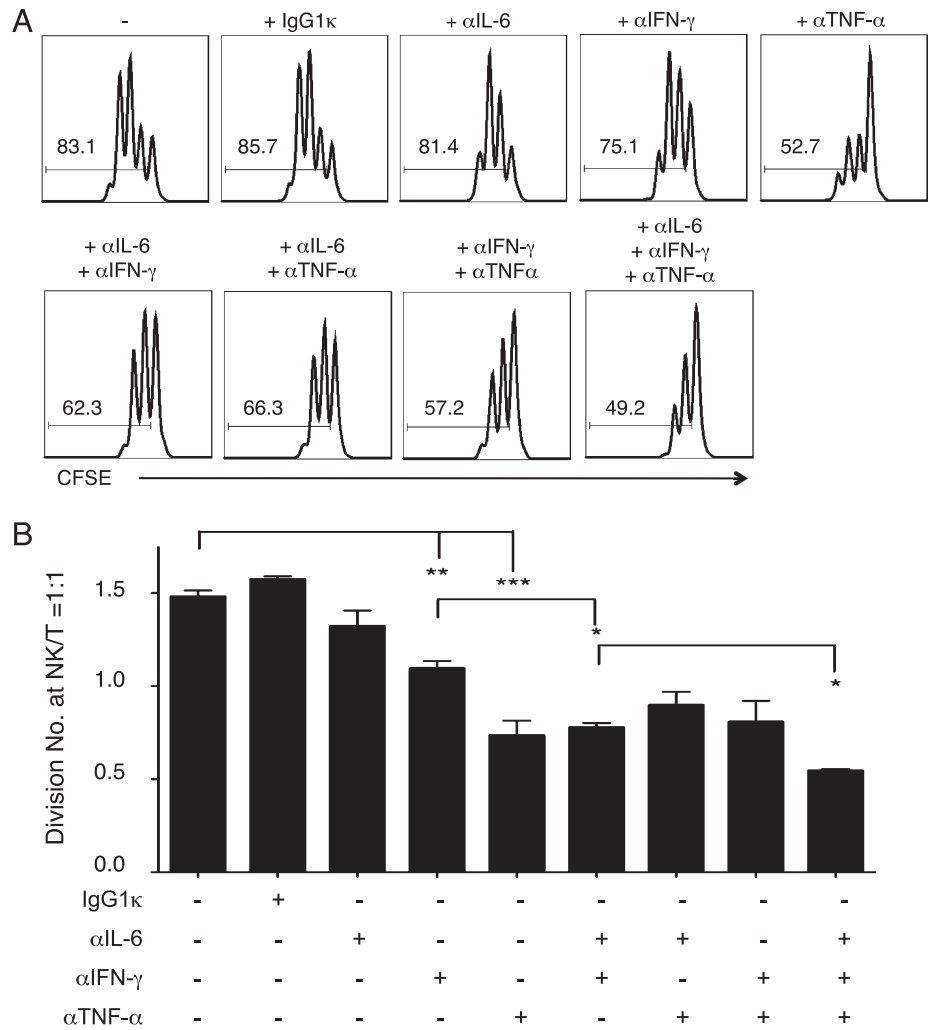


FIGURE 6. Neutralizing IFN- γ , TNF- α , and IL-6 in NK-T cell coculture ameliorated CD4⁺ T cell proliferation. NK and autologous CD4⁺ T cells were isolated from an AA haplotype licensed individual, stimulated with anti-CD3 and anti-CD28, and cocultured in 2 ng ml⁻¹ (26 IU) IL-2 for 3 d. **(A)** Histograms of CD4⁺ T cell CFSE dilution without or with the indicated neutralizing Abs. The number in each histogram indicates the percentage of cells proliferated (gated on CD4⁺CFSE⁺ cells). **(B)** Bar plot of CD4⁺ T cells division number at NK/T = 1:1 from the AA haplotype healthy individual (mean \pm SEM, n = 2–6, two-tailed Student t test, * p < 0.05, ** p < 0.005, *** p < 0.0005). More than three experiments were performed.

Besides IBD, the genetic presence of strong NK licensing KIR/ligand pairs (KIR2DL3/HLA-C1 or KIR3DL1/HLA-Bw4) also affects several other important chronic inflammatory diseases: elevated susceptibility to CD, celiac disease, spondyloarthritis, psoriatic arthritis; enhanced resolution of hepatitis C virus infection; and slower progression in HIV-1 infection (39–45). Understanding the nature of KIR contribution to disease susceptibility or protection is crucial for developing diagnostic and treatment strategies. However, biologic study of KIR-mediated disease association has been challenging because of the polymorphic composition and functions of KIR haplotypes, and the independent assortment of their cognate HLA class I ligands. By focusing on the simplified AA haplotype, which “tag” most of the inhibitory KIRs that are involved in licensing and lack most of the activating KIRs, we were able to identify KIR-mediated licensing as a major mechanism to reprogram NK cell cytokine capacity. We further showed that, in accord with the distinct cytokines produced by licensed NK cells, they have the capacity to augment CD4⁺ T cell activation and Th17 differentiation, which provides a mechanistic basis for their genetic association to IBD and other chronic inflammatory diseases.

The distinct NK cytokine program induced by KIR licensing appears to be a genetic trait independent of disease status. Thus, NK cell licensing determined cytokine program was preserved in both CD and healthy cohorts (Figs. 2–4, Supplemental Figs. 1, 2); and functionally licensed NK cell cytokines from both CD and healthy

subjects comparably augmented CD4⁺ T cell polarization (Figs. 1, 3). The exceptional breadth and proinflammatory cytokine profile of licensed NK cell is an important finding of this study, as well as the evidence that this cytokine production lowers the threshold for CD4⁺ T cell activation. Another striking finding was the selective capacity of licensed NK cell cytokines to efficiently drive IL-17A and IL-22 production. This is in part attributable to NK-derived IL-6; however, other NK-produced cytokines may also be involved, because in several culture conditions, Th17 polarization by NK cell supernatants exceeded that expected for IL-6 alone and blocking IL-6 alone did not affect Th17 differentiation (Fig. 7, Supplemental Fig. 3). Licensed NK cells synergize with IL-23 and IL-1 β to facilitate Th17 differentiation, indicating that licensed NK cell secretory products can collaborate with other cells in the local tissue compartment (dendritic cells or macrophages) to promote a more proinflammatory environment shaping CD4⁺ or CD8⁺ T cell responses (46, 47). Further studies of unlicensed and licensed cells from healthy individuals will be required to validate and refine the effects of NK licensing in Th17 differentiation.

NK cells are not abundant and are functionally heterogeneous, posing significant challenges to understand individual cell behavior. We improved and used a single cell proteomics microchip for high-throughput, highly multiplexed, tailored analysis of cytokine expression capacity of NK cells. This microfluidic platform permits detection of ~40 different protein products per individual cell and up to 1000 cells in a single experiment, and for the first

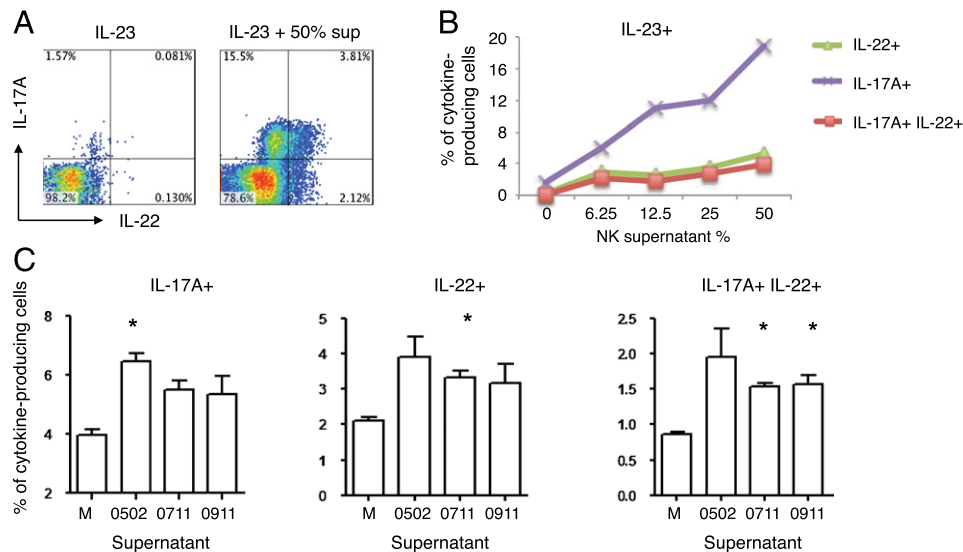


FIGURE 7. Supernatant of licensed NK cells drives human Th17 cells differentiation in vitro. Freshly isolated CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 and cultured in the presence of indicated cytokine with or without licensed NK cell supernatants for 6–7 d. CD4⁺ T cells were expanded for another 6–7 d in 2 ng·ml⁻¹ (26 IU) IL-2 with the same condition provided for priming. **(A)** Two-dimensional scatter plot of IL-17A and IL-22 intracellular production under the conditions indicated (gated on CD4⁺ cells). Numbers in each quadrant represent the percentage of cell in that quadrant. **(B)** Line plot of the abundances of IL-22⁺ (green triangle), IL-17A⁺ (purple cross), and IL-17A⁺IL-22⁺ (red square) populations at different amounts of NK supernatant. This result is representative of three independent experiments. **(C)** Bar plot of the percentages of IL-17A⁺ (left panel), IL-22⁺ (middle panel), and IL-17A⁺IL-22⁺ (right panel) CD4⁺ T cells after differentiating with 50% NK cell supernatants from three licensed healthy donors. M stands for media with the same amount of IL-2 used for NK 3-d culture. All assays have been supplemented with 50 ng·ml⁻¹ IL-23 ($n = 2$, two-tailed Student t test, $*p < 0.05$) (0502 and 0911 are AA haplotype, 0711 has an extra KIR2DL2).

time, to our knowledge, allows us to identify the striking distinctions between licensed and unlicensed NK cell subsets, and the commonality within each subset.

Finally, as the pathogenesis of CD is ultimately driven by intestinal lymphocytes; it is desirable to investigate properties of licensed NK cells resident in the gastrointestinal tract. However, the practicalities of such a study are quite difficult. Less than 10% of patients are genetically informative (KIR AA haplotype with HLA-C1/C1 genotypes); and even at a major IBD clinical center, intestinal resections from 10 patients would require 2 y to accrue. The alternative of colonoscopic biopsy sampling is also technically unsuitable. Because of the low abundance of CD3⁺CD56⁺ NK cells in the intraepithelial and lamina propria compartments [~ 18.5 and $\sim 10\%$, respectively (48, 49)], and low cellular yield per biopsy [1–2 million lymphocytes per 2-mm² biopsy (50)], ~ 20 biopsies from each patient are required for a minimal experiment, which is beyond the number permitted for research sampling. We also note that IBD is a systemic disease with $>40\%$ of IBD patients having extraintestinal manifestations. In this context, the study of NK cells from peripheral compartments is relevant to IBD biology.

Bridging the “gene to biology” and “bench to bedside” divides is one of the major challenges currently facing researchers. Our study addresses this challenge through mechanistic finding of the proinflammatory role of licensed NK cells on adaptive immunity. This offers a fresh biologic diagram accounting for the impact of KIR-HLA genetics on IBD and other chronic inflammatory diseases.

Disclosures

The authors have no financial conflicts of interest.

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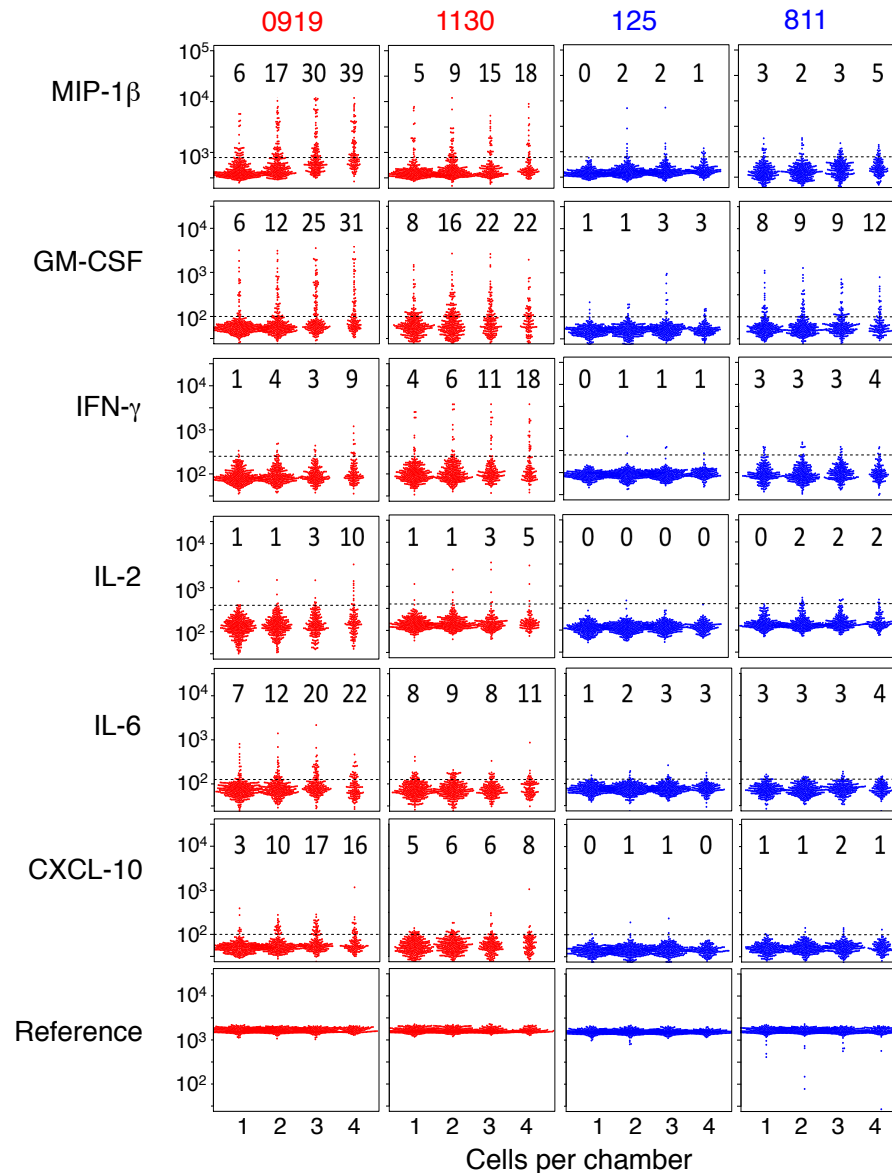
Supplementary Tables

Table S1. Hierarchy in the strength of NK cell education (adapted from ^{1,2})

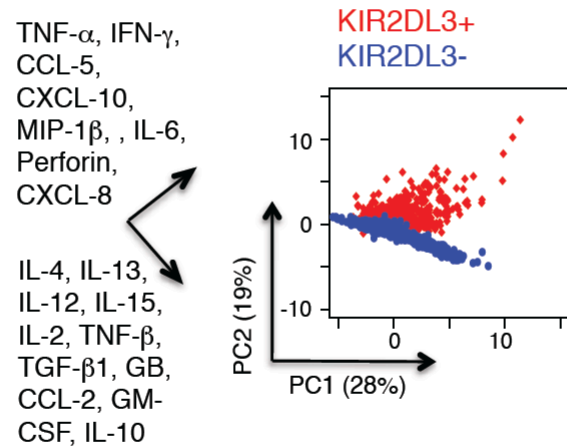
KIR	HLA	Level of Response
2DL3	Cw*07 (C1)	++++
3DL1 ^{high}	Bw4 ^{strong}	++++
2DL3	Cw*12	+++
2DL3	C1 + B*46	+++
2DL1 non*004	Cw2*02,4,5,6,15 (C2)	++
3DL1*007	Bw4 ^{strong}	++
NKG2A	HLA-E	++
2DL1*004	Cw2*02,4,5,6,15 (C2)	+
2DL3	Cw*01,3,8 (C1), 1404	+
3DL1 ^{high}	B*27	+
3DL1 ^{high}	A*24	+
2DL1	Cw*01,3,8, (C1), 1404	-
2DL3	Cw*1402	-
2DL3	Cw2*02,4,5,6,15 (C2)	-
3DL1 ^{high}	B*13	-
3DL1 ^{high}	B*37	-
3DS1	Bw4 ^{strong}	-
3DL2	A*3,11	-

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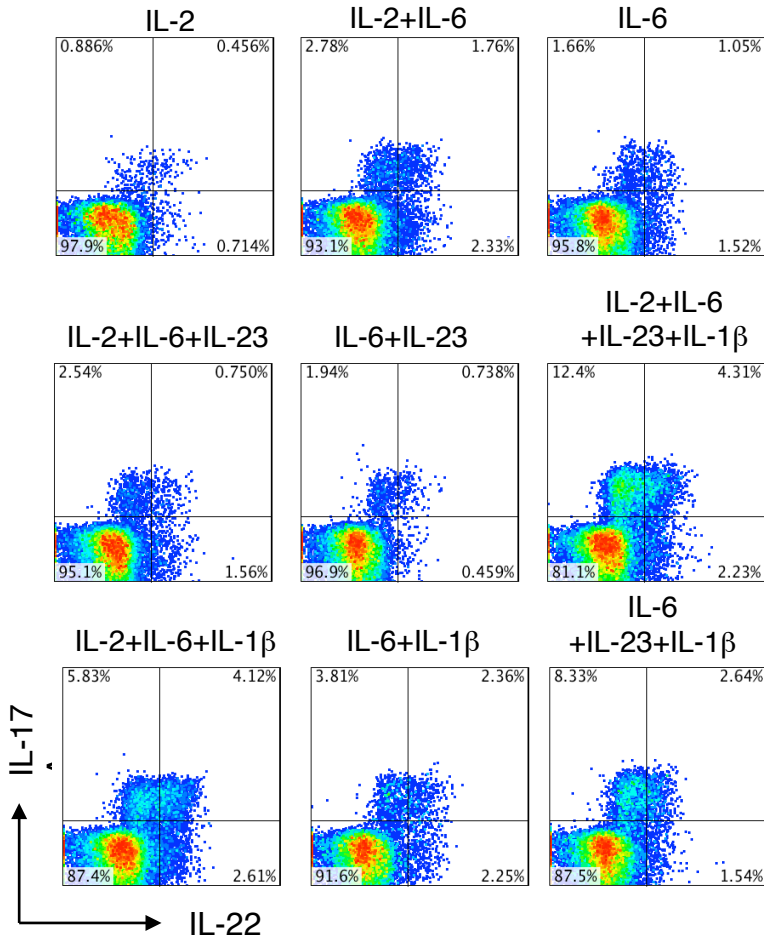
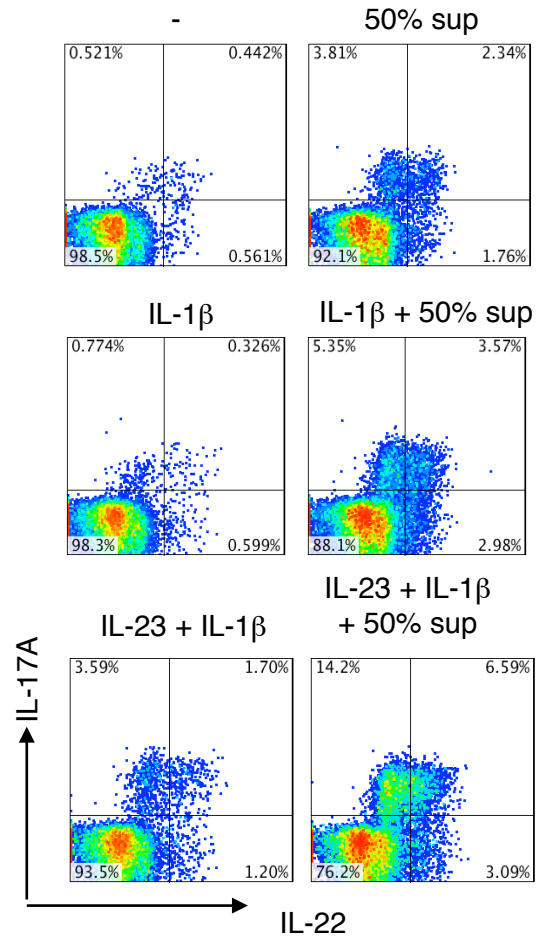
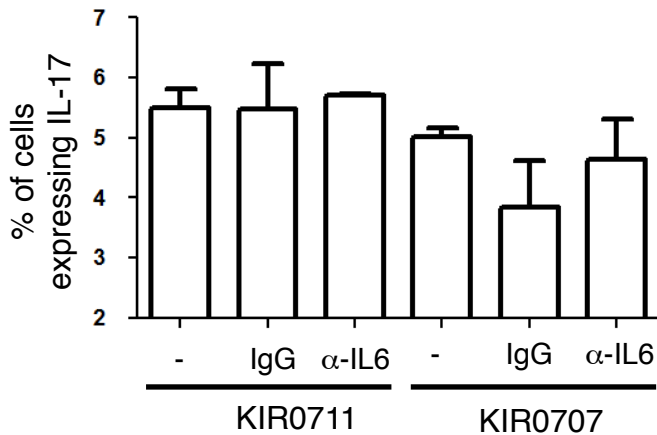
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Supplementary Figure 1. Univariate comparison of NK cell secretions from *HLA-C1C1* and *HLA-C2+* CD patient using SCBC. MIP-1 β , GM-CSF, IFN- γ , IL-2, IL-6, CXCL-10, and reference signals from 1-4 cell SCBC microchambers from *HLA-C1C1* (#0919, #1130, red) and *HLA-C2+* (#0125, #811, blue) CD patients.



Supplementary Figure 2. KIR2DL3⁺ NK cells from an AA haplotype individual display higher effector protein production capacity than KIR2DL3⁻ NK cells. PCA analysis of NK secretion profiles from KIR2DL3⁺ and KIR2DL3⁻ NK subsets. Percentage of variation explained by each component is shown in parentheses. The factors for each component are indicated left of the plot.

A**B****C**

Supplementary Figure 3. NK cell supernatant from *HLA-C1C1* healthy donor facilitated *in vitro* T_H17 differentiation. (A) *in vitro* T_H17 differentiation in various cytokine combinations. Scatter plot of IL-17A and IFN- γ intracellular production from CD4⁺ T cells at D14 after differentiation with or without IL-2 at 2 ng mL⁻¹, in the presence or absence of IL-1 β , IL-23, or IL-6, at 50 ng mL⁻¹, as

indicated. (B) Scatter plot of IL-17A and IL-22 intracellular production from CD4⁺ T cells at D14 after differentiation with IL-1 β or IL-23 and IL-1 β , at concentration of 50 ng mL⁻¹, with or without the addition of NK cell supernatant. Numbers in each quadrant represents the percentage of cell in that section. (C) Effect of IL-6 depletion (5ug/mL) from 2 *HLA-C1C1* healthy donor NK cell supernatants in T_H17 differentiation *in vitro*.