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More than a License to Kill: Immune Regulation of Crohn's Disease by Natural Killer Cell and Its Clinical Implication

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More than a License to Kill:

Immune Regulation of Crohn's Disease by Natural Killer Cell and Its Clinical Implication

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular and Medical Pharmacology

by

Lin Lin

2013

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ABSTRACT OF THE DISSERTATION

More than a License to Kill:

Immune Regulation of Crohn's Disease by Natural Killer Cell and Its Clinical Implication

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Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2013

Professor Jonathan Braun, Chair

Inflammatory bowel disease (IBD) is a group of chronic inflammatory conditions in the gut. A major form of IBD is Crohn's disease (CD). Natural Killer cells are members of the innate immune system mainly known for their cytolytic abilities against infected or tumor cells. They are divided into distinct subsets comprised of licensed and unlicensed cells. NK cells are programmed at a genetic level to express discriminating Killer Immunoglobulin Receptors (KIRs) in humans and Ly49 receptors in mice. The engagement of these receptors by their cognate human leukocyte antigen (HLA) ligands induces differentiation of NK cells into a licensed state, which has functional properties distinct from the unlicensed states. It has been identified that the genetic presence of KIR2DL2/3 in the context of its ligand HLA-C1 is a risk factor for CD, yet the cellular

mechanism of this genetic contribution and its contribution to immune-mediated colitis is unknown.

We used a co-culture assay to study if peripheral NK cells from CD patients with KIR2DL2/3 and HLA-C1, a genotype permits strong NK cell licensing, have a differential impact on $CD4^+$ T cell proliferation than patients without this genotype. Using co-culture assays, flow cytometry, and a microfluidic platform for cytokine analysis, we assessed the role of KIR-HLA genetics on the immunologic functions of NK cells. To identify if KIR-HLA genetics can be used to predict thiopurine responsiveness, we stratified an independent pediatric inflammatory bowel disease cohort into licensed and unlicensed subsets, and compared the percentages of responders in the two subsets.

NK cells from CD patients with strong licensing genotype are more potent in promoting $CD4^+$ T cell proliferation than those from patients with intermediate or low licensing genotype ($p<0.005$ and $p<0.0005$ respectively). The effect of licensed NK cell is independent on direct contact. Multiplexed analysis of bulk media and single cell NK cytokine profiles established that licensed NK cells produced higher levels pro-inflammatory cytokines including interferon-γ, tumor necrosis factor-α, and chemokines, including C-C motif ligand-5 and macrophage inflammatory protein-1β. Licensed NK cell supernatant also dramatically promotes the differentiation of T_H17 cells, a signature $CD4^+$ T helper subset in CD. Pediatric patients with licensing (strong and intermediate) genotype have a higher responder rate (64.7%) to thiopurines than those with low licensing genotype (20.0%).

Using similar methods, we demonstrated that murine licensed NK cells augment $CD4^+$ T cell activation not only by increased secretion of proinflammatory mediators

such as IL-6 and TNF-α, but also through contact-dependent mechanism, which is different from the solely cytokine-mediated mechanism seen in human licensed NK cells.

In conclusion, NK cell licensing mediated by KIR2DL2/3 and HLA-C1 elicits a novel cytokine program that induces pro-inflammatory $CD4^+$ T cells activation and Th17 cell differentiation, thereby providing a pharmacogenomic tool for predicting responders to thiopurine treatment in inflammatory bowel diseases. Murine and human NK cell licensing share similar features, and its role in immune colitis awaits further investigation.

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DEDICATION

To my loving and supporting parents

Qiuping Zheng and Jiazhen Lin

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LIST OF ABBREIVIATIONS

7-AAD	7-amino actinomycin D
AU	Arbitrary Unit
αGalCer	(2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol
CCL-2	C-C chemokine Ligand type -2
CCL-5	C-C chemokine Ligand type -5
CD	Crohn's disease
CD3	cluster of differentiation 3
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CD28	cluster of differentiation 28
CD56	cluster of differentiation 56
CD244	cluster of differentiation 244
CD252	cluster of differentiation 252
CFSE	Carboxyfluorescein succinimidyl ester
CHORI	children’s hospital oakland research institute
CSMC	Cedars Sinai Medical Center
CXCL-8	C-X-C motif chemokine 8
CXCL-10	C-X-C motif chemokine 10
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting

FBS	Fetal Bovine Serum
bFGF	basic fibroblast growth factor
GB	Granzyme B
GM-CSF	granulocyte-macrophage colony stimulating factor
HC	Hierarchical Clustering
HCLE	human corneal limbal epithelial
HIF	hypoxia-inducible factors
HIPPA	health insurance portability and accountability act
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HUVEC	human μmbilical vein endothelial cell
IBC	institutional biosafety committee
IBD	inflammatory bowel diseases
IEL	intraepithelial lymphocyte
IFN-γ	interferon-gamma
IL	interleukin
ILC	innate lymphoid cell
IRB	institutional review board
ITN	immune tolerance network
KIR	killer cell immunoglobulin-like receptor
KSFM	keratinocyte serum-free medium
LI	large intestine
LPL	lamina propria lymphocyte

LRC	leukocyte receptor complex
LTi	Lymphoid tissue inducer
MHC	Major Histocompatibility Complex
MIP-1β	Macrophage inflammatory protein-1beta
MLN	mesenteric lymph nodes
MS	multiple sclerosis
NK	natural killer
NO	nitric oxide
NV	neovascularization
O_2^-	superoxide anion
$ONOO^-$	peroxynitrite
OUT	operational taxonomic units
OX40L	OX40 ligand
rpm	round per minute
PBMC	peripheral blood mononuclear cell
PCA	principal component analysis
QIIME	Quantitative Insights Into Microbial Ecology
RF	restricted flora
PMA	Phorbol 12-myristate 13-acetate
PP	peyer's patch
RT-PCR	reverse transcription polymerase chain reaction
SCBC	single cell barcode chip
SI	small intestine

SIN-1	3-morpholinosyndnomine
SPF	specific pathogen-free
TGF-β1	Transforming growth factor beta 1
Th	T helper
TNF-α	tumor necrosis factor alpha
TNF-β	tumor necrosis factor beta
UC	ulcerative colitis
VEGF	vascular endothelial growth factor

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Ashki, N., Chan, A., Qin, Y., Kiyohara, M., **Lin, L**., Wadehra, M., Braun, J., & Gordon, L. Peroxynitrite up-regulates angiogenic factors, VEGF-A, bFGF, and HIF-1a, in human Corneal limbial epithelial cells, 2013, *submitted*

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Lin, L. Genetically-determined cytokine programs of human NK cells uncover a novel mechanism of NK cells and Killer cell IgG-like Receptor (KIR) genes in Crohn's Disease (CD) susceptibility. FOCiS, Boston, MA, 2013

Lin, L. KIR-mediated NK education: Potential mechanism for KIR-associated IBD susceptibility. FOCiS, 2012, Vancouver, Canada

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Lin, L. KIR-mediated licensing induces a broad change in natural killer functional repertoire. Molecular & Medical Pharmacology retreat, 2012, Huntington Beach, CA

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CHAPTER 1

Another Earth: Innate Lymphoid Cells and Intestinal Inflammation

Introduction

In the ever-weird world of immunity, a new category of lymphocytes has emerged, innate lymphoid cells (ILC). Prominent in the intestinal mucosa, they sense environmental rather than antigenic cues and deliver the full scope of inflammatory functions a magnitude faster than adaptive B, $CD4^+$, or $CD8^+$ T cells. The discovery of functionally diverse ILC subsets suggests the presence of an innate version of cytotoxic, Th1, Th2, Th17, and Th22 immunity. From a distance, these 2 sides of immunity are distinct in detail, but common in function and meaning (Figure 1-1). We may need to rethink how much immune control occurs at the innate level and the new opportunities this offers for therapeutic manipulation of infection and inflammatory disease.

Results

ILC and What They Do

ILC are a distinct family of lymphocytes from B or T cells, because their developmental pathway is unique and they are equipped environmental sensors rather than antigen-specific receptors. Localized to the mucosa and related stromal tissues, ILC bear a cellular physiology designed for rapid, innate protective responses.[1] The first identified ILC was the natural killer (NK) cell, based on its innate (antigen-receptor independent) detection of virally infected and stressed cells, prompt and potent cytolytic activity, and secrete of Th1 proinflammatory cytokines (notably interferon- γ; termed conventional NK). Thymic-derived NK cells may exclusively produce Th1 cytokines, and hence have been termed ILC1. The roles of NK cells in gastrointestinal diseases have been widely studied, most notably as potent and early participants in recognition of viral and intracellular pathogen infections.[2] NK and ILC1 cells are under tight control by the large family of activating and inhibiting killer immunoglobulin- like receptors (KIR), which modulate their responsiveness to mucosal cells bearing cognate KIR ligands (class 1 major histocompatibility complex [MHC] genes), whose display in turn is tuned by environmental cues.[3] The activity of NK cells and ILC1 is divergent among individuals, in part because of the exceptional combinatorial genetic polymorphism of KIRs and MHCs between individuals. Hence, resistance to important viral pathogens such as cytomegalovirus, hepatitis C, and human immunodeficiency virus show a strong association with select stimulatory or inhibitory KIRs and their cognate MHC loci.[3-8]

NK and ILC1 paradoxically seem to be protective in inflammatory bowel disease, but are deleterious in celiac disease. NK cells have reduced cytotoxic activity in IBD

patients, [9] and there is genetic association of KIR and MHC alleles with IBD disease risk.[10,11] In contrast, NK-like functions are a prominent, disease-associated process in celiac disease, although the relative contributions of NK and ILC1, compared with the innate-like function of adaptive lymphocytes are still being dissected.[12]

Lymphoid tissue inducer (LTi) cells are critical organizers of lymphoid tissue formation and adaptive immunity, through their activation of select stromal cells, chemokine-mediated recruitment of hemopoietic cells, and B-cell differentiation for immunoglobulin A production.[13,14] Upon mucosal bacterial infection and attendant mucosal myeloid IL-23, LTi cells are the major early producers of interleukin (IL)-17 and IL-22, and hence important for protective neutrophil recruitment (IL-17) and epithelial defense and survival mechanisms (IL-22).[15]

ILC restricted to production of IL-17 or IL-22 (ILC17 and ILC22, respectively) overlap with LTi in terms of developmental requirements and anatomic distribution. Indeed, it is possible that, like ILC1 and NK, ILC17 and ILC22 may be alternate differentiation states of the LTi sublineage. ILC17 accumulate at sites of inflammation in experimental microbial and immune-mediated colitis, and infection associated IL-23 production stimulates ILC17 to produce large amount of IL-17 and interferon-γ.[16] Human ILC17 cells are overrepresented in the colon and ileum of Crohn's disease patients, are a major source of tissue IL-17, and are critical to pathological outcome.[17] ILC22 are present in intestine, mainly residing in small intestine lamina propria. Like NK and ILC-1 cells, ILC22 development requires IL2 and IL-15 signaling,[18] but they also require microbial flora for differentiation; ILC22 are deficient in germ-free mice, but are elicited after colonization with normal microflora.

ILC22 in human also are primarily located in mucosa-associated lymphoid tissue (tonsils and Peyer's patches), where they secrete IL-22 and IL-26 in response to IL-23. They are equipped for homing and adherence to epithelial cells, and through this interaction induce epithelial IL-10, a powerful anti-inflammatory cytokine. Thus, human ILC22 seem to be specialized for restoring epithelial recovery after infection.[19]

Host protection against parasitic infections is a specialized feature of TH2 adaptive immunity, but the innate immune equivalent was not recognized until the recent discovery of TH2-like ILC (ILC2). Defined by their production IL-5 and IL-13, they reside in mesenteric lymph nodes and adipose tissue ("nuocytes"), as well as in the peritoneal cavity ("natural helper" cells). Upon helminthic infection, mast cell IL-25 and epithelial IL-33 directly activate ILC2 to expand and produce mediators required for early eosinophil recruitment and mucosal processes for worm expulsion. [20,21]

Discussion

The recognition of ILC has revealed a parallel world of adaptive and innate lymphocytes proficient in each of the immune modes of microbial defense and inflammatory regulation. These two worlds share the same activation mechanisms and responsiveness to chemokine and cytokines. So, either may be reflected in genome wide association studies and targets of current immunologic therapies observed in diseases like viral hepatitis and inflammatory bowel disease. We are thus confronted with conundrums. How much of our thinking about adaptive immunity in disease pathogenesis is instead operating at the innate level? Can we create useful analytic and genetic methods to better distinguish which parallel universe is at play for an individual patients' disease risk and monitor the nature of disease activity? And, can we better define the sensors and regulators for ILC that could provide targets for their therapeutic manipulation? We may not need another earth, but now that we have one, let's embrace the wonder and opportunity.

Figures and Tables

Figure 1-1

Figure 1-1. Visible Earth.

NASA Goddard Space Flight Center images by Reto Stöckli and Robert Simmon.

Available at: http://visibleearth.nasa.gov/view_rec.php?id=2429.

CHAPTER 2

KIR-HLA Genes Modify the Action of NK Cells in Crohn's Disease and Predict the Responsiveness to Thiopurine Therapy

Abstract

Background

Crohn's disease (CD) is a group of chronic inflammatory conditions in the gut, a major form of inflammatory bowel diseases. It has been identified that the killer cell immunoglobulin-like receptor gene (KIR)2DL2/3 of natural killer (NK) cells in the context of its ligand human leukocyte antigen (HLA)-C1 is a risk factor for CD, yet the cellular mechanism of this genetic contribution is unknown.

Methods

We used a co-culture assay to study if peripheral NK cells from CD patients with KIR2DL2/3 and HLA-C1, a genotype permits strong NK cell licensing, have a differential impact on $CD4^+$ T cell proliferation than patients without this genotype. Using co-culture assays, flow cytometry, and a microfluidic platform for cytokine analysis, we assessed the role of KIR-HLA genetics on the immunologic impact of NK cells on $CD4^+$ T cell proliferation and differentiation. To identify if KIR-HLA genetics can be used to predict thiopurine responsiveness, we stratified an independent pediatric inflammatory bowel disease cohort into licensed and unlicensed subsets, and compared the percentage of responders in the two subsets.

Results

NK cells from CD patients with strong licensing genotype are more potent in promoting $CD4^+$ T cell proliferation than those from patients with intermediate or low licensing genotype ($p<0.005$ and $p<0.0005$ respectively). The effect of the licensed NK cells is independent on direct contact. Multiplexed bulk media and single cell analysis of cytokine profiles established that licensed NK cells produced higher levels pro-

inflammatory cytokines including interferon-γ, tumor necrosis factor-α, and chemokines, including C-C motif ligand-5 and macrophage inflammatory protein-1. Licensed NK cell supernatant also dramatically promotes the differentiation of T_H17 cells, a signature $CD4^+$ T helper subset in CD. Pediatric patients with licensing (strong and intermediate) genotype have a higher responder rate (64.7%) to thiopurine than those with low licensing genotype (20.0%).

Conclusion

NK cell licensing mediated by KIR2DL2/3 and HLA-C1 elicits a novel cytokine program that induces pro-inflammatory $CD4^+$ T cells activation and Th17 cell differentiation, thereby providing a pharmacogenomic tool for predicting responders to thiopurine treatment in inflammatory bowel diseases.

Introduction

NK cells are components of the innate immune system primarily known for cytolytic targeting of tumor cells and virally infected cells. However, the roles and functional proficiencies of NK cell subpopulations are complex, including their involvement in chronic inflammatory diseases. [2,22-24]

There have been great strides in identifying genetic susceptibility loci for chronic inflammatory and autoimmune diseases, but a significant task remains to identify the functional consequences of these disease-associated variants. [25] This task is framed by the functional networks emerging in disease-associated genes and the combinatorial features of these networks in the host phenotype. [26] One important example is the genetic combination of killer immunoglobulin-like receptors (KIR) with their respective HLA class I ligands, which has been associated with multiple autoimmune disorders, infectious diseases, and cancers. [27,28] However, the functional mechanisms accounting for these disease associations, particularly for the inhibitory class of KIRs, are poorly understood. One challenge is that the KIR gene family cannot be systemically studied in model systems, as they are not present in the rodent genome. The other is that the HLA and KIR gene families are functionally polymorphic, and each KIR has a selective affinity for specific alleles of individual class I HLA genes. The KIR gene family is encoded in a 100-200 kb region of leukocyte receptor complex (LRC) on chromosome 19q13.4, comprised of 14 functional genes (seven activating, six inhibitory, one bi-functional) and 2 pseudogenes. [29] 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-B allotypes with the serologically defined Bw4 motif; and some KIRs (2DL4 and 3DL3) lack known ligands. [27,29] Accordingly, the representation of individual HLA and KIR genes in an individual haplotype is quite heterogeneous for functional pairings of cognate HLA and KIR genes, which confounds studies of their biologic function.

KIR genes are predominantly expressed by NK cells, and are one element of a repertoire of cell surface receptors controlling NK cell activation, proliferation, and effector functions that mediate surveillance and host defense for microbial infection and malignancy. [30,31] The involvement of NK cells in various chronic inflammatory diseases has also been reported, but their roles are less well understood. [27,32-35] For example, there is evidence for NK cells either elevating or attenuating disease penetrance in different murine models of immune colitis. [36,37] The present study was prompted by the elevated genetic susceptibility for Crohn's disease in patients bearing the inhibitory KIR2DL2 and KIR2DL3 with its cognate ligand HLA-C1. [10,11,38]

We were puzzled by this association, since NK-target interaction via inhibitory KIR ligation and self-MHC signals an immunoreceptor tyrosine-based inhibitory motif (ITIM)-mediated suppression of NK effector function that averts auto-aggressive tissue destruction. [23,39] One potential explanation of this association is that a decreased NK-dependent immune response to intestinal microbes may contribute to the microbial dysbiosis involved in Crohn's disease pathogenesis. However, thus far no such role for NK cells has been uncovered. A second explanation is the paradoxical function of inhibitory KIRs during NK differentiation: inhibitory KIR ligation induces NK cell licensing, a maturational process resulting in the acquisition of efficient target killing and

IFN-γ-induction proficiencies. [39,40] The influence of NK licensing on other aspects of NK cell function remains largely unknown, including its role in chronic inflammatory settings, its impact on adaptive immune response, and the scope of licensing-related cytokine and chemokine production. [41]

Therefore, we aimed to investigate the potential role of NK licensing in the KIR associated Crohn's disease susceptibility. Here 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 the differentiation of T_H17 cells, a signature $CD4^+$ T helper subset in CD. Multiplexed cytokine study of two independent CD and MS disease cohorts demonstrated that genetically licensed and unlicensed NK cells exhibit consistent and distinct cytokine profiles, with licensed NK cells distinguished by high-output, pro-inflammatory, poly-cytokine expression. Selected cytokines among this output account for the unique capacity of licensed NK cells to efficiently augment antigenic $CD4^+$ T cell proliferation and T_H17 polarization.

Thiopurine drugs including 6-mercaptopurine (6-MP) and azathioprine (AZA) have a long-standing, proven efficacy to induce steroid-free remission (~30%) in CD patients and maintain remission (50%) in Ulcerative colitis (UC).[42,43] But these agents have toxicity, and their use supplant other treatment choices until their efficacy or lack thereof is established clinically. The challenge is to identify which individuals may benefit from this therapy, since the exact cellular target of thiopurine therapies is not yet established, and there isn't a strategy to identify patients who would likely respond.

Thiopurines act by competitively inhibiting Rac1 function in lymphocytes, [44] and is known to preferentially eliminate peripheral and lamina propria NK cells and diminish their function. [45,46] Our hypothesis is that thiopurine is therapeutic in IBD by depleting NK cells, and this depletion is of clinical utility only in patients with licensed NK cells. Our prediction is that patients with licensing genotype are the responsive clinical subset.

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 UCLA. CD patient, previously genotyped for HLA and KIR[10], were consented and called back by CSMC. 455 out of 1306 CD patients are AA haplotype, 28 were consented for call-back blood donation, and 20 samples were collected. Healthy donors were recruited at UCLA Clinical & Translational Research Laboratory, and genotyped by the UCLA Immunogenetics Center. The multiple sclerosis (MS) cohort of 50 frozen PBMC samples were provided by Immune Tolerance Network (ITN), and KIR and HLA typing were performed by Children's Hospital Oakland Research Institute (CHORI). A complete clinical sample process and assay pipeline is provided (Figure 2-1).

Cell isolation

PBMCs were isolated by Ficoll-Plaque (GE Healthcare, Chalfont St Giles, England) density gradient centrifugation. Human NK cells were purified either from whole blood using the RosetteSep Human NK cell enrichment Cocktail, or from PBMC using Human NK cell negative selection kit (StemCell Technologies, Vancouver, BC, Canada). Human Treg depleted T cells were purified from PBMC using Human T cell enrichment kit and CD25 positive selection kit, total CD4 T cells were purified from PBMC using Human T cell enrichment kit and CD4 positive selection kit (StemCell Technologies, Vancouver, BC, Canada). The purity of isolated NK cells and T cells were checked to be above 90%.

NK-T cell co-culture and blocking assays

Before co-culture, round bottom 96-well plates were coated with anti-CD3/CD28 antibody (R&D Systems, Minneapolis, MN) in PBS at 1.5μg ml^{-1} at room temperature for 2 hours, or at 0.5μg ml^{-1} at 4ºC overnight. T cells were stained with 0.2μM CFSE (Invitrogen, Carlsbad, CA), and co-cultured with NK cells for 72 hours in 96-well plates at 1 x 10^6 cells ml^{-1} in presence of 2ng ml^{-1} (26 I.U) IL-2 with complete RPMI 1640 medium (recipe provided above). Blocking antibodies for OX40 ligand and 2B4 (CD252 and CD244, R&D Systems, Minneapolis, MN) were added to the co-culture at concentration of 10μg ml^{-1}. Neutralizing antibodies, for IL-6, IFN-γ, TNF-α, and isotype control mouse IgG1κ (ebioscience, San Diego, CA), and their combinations were added to the co-culture at 1.25μg ml^{-1}. The recombinant cytokines IL-6, IFN-γ, and TNF-α (R&D Systems, Minneapolis, MN) was each added to a final concentration of 20 ng ml^{-1}, comparable to the concentration measured for these cytokines in NK cell 72-hour 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.

T_H17 differentiation assay

Before culturing, round bottom 96-well plates were coated with 1μg ml^{-1} anti-CD3 (R&D Systems, Minneapolis, MN) in PBS for 2 hours at room temperature, and washed with 5% human AB Serum RPMI media (Lonza, Rockland, ME). Total $CD4^+$ T cells were

purified and stimulated with 0.2µg 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 re-suspended, washed once with media, and expanded with 2ng ml^{-1} (26 I.U) IL-2 plus the same conditions provided for priming. At Day 14, the cells were stimulated with PMA/Ionomycin and Brefeldin A at 1uL per well for 5 hours. 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 assays

CD and MS frozen PBMCs were thawed and recovered overnight, and NK cells were isolated using Human NK cell negative selection kit (StemCell Technologies, Vancouver, BC, Canada). NK cells were cultured for 72 hours in round-bottom 96-well plate at 1 x 10^6 cells ml^{-1} in 2ng ml^{-1} (26 I.U) IL-2 with complete RPMI 1640 medium, containing 10% Fetal Bovine Serum (FBS), 100 I.U ml^{-1} penicillin 100 µg ml^{-1} streptomycin, 10mM HEPES buffer, 2 mM glutamine (Cellgro, Manassas, VA), and 5 x 10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). Then CD media samples were collected, stored at -80^oC. Before analysis, samples were thawed, concentrated 4 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 antibody panel was chosen to incorporate non-redundant secretions detectable at NK 72-hour culture. (Supplementary Figure 2)

Single cell multiplex cytokine analysis

Frozen aliquots of PBMC were thawed and recovered overnight. Bulk NK cells were purified using 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 upon isolating individual or a small number of cells into thousands of ~600 picoliter volume microchambers, with each chamber equipped with a miniaturized antibody array. The chips used here permitted the simultaneous measurement of 19 protein markers in each microchamber (Supplementary Table 4). After loading onto the SCBC, the cells were stimulated with 5ng ml^{-1} PMA and 500ng ml^{-1} Ionomycin for 12 hours at 37ºC, 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.

Antibodies

The following antibodies and cell tracer were used staining for flow analysis: FITC-conjugated anti-CD158b (BD Biosciences, San Jose, CA), anti-IFN-γ (ebioscience, San Diego, CA); CFSE; PE-conjugated anti-IFN-γ (BD Biosciences, San Jose, CA), anti-TNF-α, anti-IL-22, anti-Granzyme B (ebioscience, San Diego, CA), anti-GM-CSF (R&D Systems, Minneapolis, MN); PerCP-conjugated anti-CD3 (BD Biosciences, San Jose, CA); APC-conjugated anti-CD158a (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD4 (BD Biosciences, San Jose, CA), anti-IL-17A

(ebioscience, San Diego, CA); strepavidin-PerCP; PE-Cy7-conjugated anti-CD56, anti-CD14 (BD Biosciences, San Jose, CA), Vioblue-conjugated anti-3DL1 (Miltenyi Biotec, Bergisch Gladbach, Germany), eFluor 650NC-conjugated anti-CD3 (ebioscience, San Diego, CA). anti-mouse IgGκ /Negative Control Compensation Particles. The use of antibody for staining was performed as per the manufacturer's instructions with proper titrations. Antibodies used for cytokine assays are IL-2, IL-6, IL-10, IL-15, IL-13, CCL-4 (MIP-1β), 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, perforin (eBiosciences, San Diego, CA).

Flow Cytometry and Cell sorting

Phenotypic analysis of PBMC was performed using flow cytometry after staining of cells with fluorescence dye–conjugated antibodies. Labeled cells were analyzed with a FACSCalibur flow cytometer using CellQuest software, or LSR II (BD Biosciences, San Jose, CA) using FACSDiva software (BD Biosciences, San Jose, CA) at UCLA Flow Cytometry Core, and data analysis was performed using FlowJo (Tree Star Inc., 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, San Jose, CA).

Statistical analysis and data access

Student's two-tailed unpaired t test was used; P values of less than 0.05 were regarded as significant. Association analyses were performed using contingency table

testing and a standard chi-square measure. All cytokine data were normalized before biostatistical analysis. GraphPad Prism (San Diego, CA) was used for statistical analysis and graphing. Principal Component Analysis (PCA), Hierarchical Clustering (HC), box-plot and scatter-plot analysis were performed in R package using custom-written codes. Microchip data from this study is available from the website (http://www.its.caltech.edu/~heathgrp/).

Results

Coexistence of KIR2DL3 and HLA-C1 genes predisposes AA patients for susceptibility to Crohn's Disease.

Mechanistic studies of human NK licensing are challenging due to the complex composition of KIR-HLA combinations [29], and the conflicting roles inhibitory and activating KIRs play in licensing. Therefore, we studied individuals homozygous for the KIR A haplotype (Table 2-1), 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 [29,47]. Different inhibitory KIR-HLA class I ligand pairs confer various levels of strength for NK licensing (Table 2-2). [48] The most potent pair is KIR2DL3/HLA-C1, and the second strongest pair is KIR3DL1/HLA-Bw4. We hypothesized that strong NK licensing confers a trait that enables NK cells to induce CD pathogenesis. To determine if KIR2DL3/HLA-C1 is genetically associated with increased susceptibility to CD in AA haplotype individuals, we analyzed the distribution of C1/C2 and Bw4/Bw6 allotypes in an AA haplotype subpopulation of our CD cohort. [10] Among the 455 KIR haplotype AA CD patients studied, the distribution of C1C1, C1C2, and C2C2 in Bw6/Bw6 was significantly disproportionate to that in Bw4/Bw6 and Bw4/Bw4 patients (Chi square test: *** $p < 0.0005$ in Table 2-3). Hence, there was an enrichment of C1C1 in Bw6/Bw6; and *vice versa*, Bw6/Bw6 was enriched in C1C1. This analysis indicated that the C1C1 Bw6/Bw6 genotype predicted genetic predisposition to CD in AA haplotype individuals.

Licensed NK cells strongly promote the proliferation of autologous $CD4^+$ T cells.

Pro-inflammatory $CD4^+$ helper T cells are the main effectors in induction and perpetuation of intestinal inflammation. [49,50] As a major cellular component of innate immunity, NK cells demonstrably cross-talk with the adaptive immunity arm. [24,47,51-53] Since NK cells can stimulate or inhibit T cell activation *via* multiple mechanisms, [54-57] we first asked if strongly and weakly licensed NK cells from CD patients could differentially modulate T cell proliferation in *in vitro* co-cultures. We isolated peripheral blood NK cells and autologous T cells from CD patients, co-cultured them in the presence of immobilized anti-CD3/CD28 and IL-2 at 2ng mL^{-1} (26 I.U.). At day 3, $CD4^+$ T cell proliferation was measured via CFSE dilution (Figure 2-1). We found that $CD4^+$ T cell proliferation was augmented in a linearly with the number of licensed NK cells present (Figure 2-2, R^2=0.996, when proliferation is measured by the increase in the average division number at each NK:T ratio compared to that of T cell alone). This indicated that licensed NK cells augmented $CD4^+$ T cell proliferation in a dose-dependent manner. Using linearity (R^2>0.85) as a quality control criterion, 12 patient assays were selected for genetic correlation analysis. At a NK:T ratio of 1:1, C1C1 NK cells were significantly more potent than $C2^+$ NK cells in all AA haplotype patients, and Bw4/Bw4 NK cells were significantly more potent than $Bw6^+$ NK cells within the $C2^+$ subset of patients (Figure 2-3A, B). Thus, three distinct levels of NK function were observed: C1C1 $Bw6^+$ > Bw4/Bw4 > Bw6/$^+$ $C2^+$ (Figure 2-3C), and this order conforms to KIR licensing strength (Table 2-2) [48].

To assess the mechanism of $CD4^+$ T cell proliferation augmentation, we considered that NK cell interaction might involve their expression of co-stimulatory molecules, such as 2B4 and OX40 ligand. [55-57] However, augmentation was preserved

when these surface molecules were blocked with neutralizing antibodies (Figure 2-4A), indicating that the NK augmentation of $CD4^+$ T cell proliferation does not depend on the expression of 2B4 and OX40. To determine if the NK effect was contact-dependent at all, we separated NK cells from T cells using 1.0 μm pore transwells, which allows cellular communication only through soluble factors. To our surprise, augmentation was fully preserved (Figure 2-4B). These results strongly suggested that the NK augmentation of $CD4^+$ T cell proliferation was mainly mediated by soluble molecules secreted by licensed NK cells.

Genetically licensed NK cells exhibit elevated cytokine production capacity compared to unlicensed NK cells.

NK cells are capable of secreting multiple cytokines and chemokines, [41] but little is known about their scope of cytokine reprogramming by licensing. Therefore, we cultured NK cells for 72 hours under the same condition used for NK-T cell co-culture experiments, and quantitated the level of a panel of cytokines in the NK supernatant using a multiplex ELISA chip [58,59]. We initially screened for 31 candidate cytokines and found 24 of them readily detectable among different subjects, although a smaller number of cytokines were detectable in any one individual (Figure 2-5 and Table 2-4). We selected a final panel of 18 non-redundant proteins (and IL-2 in some assays) was chosen based on their secretion level, as well as their importance in NK cell biology (Table 2-4). When supernatants from strongly (C1C1) and weakly ($C2^+$) licensed CD patient NK cells were compared, we observed that C1C1 NK cells had significantly more robust production for 9 cytokines (Figure 2-6). This feature was specific to NK

cells, since the production of T cells was indistinguishable from C1C1 and $C2^+$ patients (Figure 2-7). The core difference between C1C1 and $C2^+$ NK cells resided in CCL-5, MIP-1β chemokines important for neutrophil and T cell recruitment); and, IFN-γ, TNF-α, IL-6, and IL-4 (pro-inflammatory cytokines known to play a role in CD). Hierarchical clustering and principal component analysis (PCA) showed that C1C1 and $C2^+$ patients were completely separated from each other (Figure 2-8 and Figure 2-5), demonstrating their distinct secretion capacities.

To determine if such a difference is a common feature in different chronic disease settings, we evaluated PBMC NK cells from an archival cohort of 50 MS patients previously assembled by Immune Tolerance Network (ITN). The samples were first genotyped for KIR haplotype and HLA allotypes. Because of the limiting number of C1C1 AA patients available, we didn't restrict our analysis to AA haplotype patients (Table 2-5). We observed a similar C1C1-Bw6/Bw6 enrichment among the 50 patients (Table 2-5, Chi square test not conducted due to limited sample size). NK cell supernatants were produced from aliquots of 27 of these patients with sufficient cells recoverable for multiplex ELISA analysis (>100,000 NK cells). The licensed group was operationally defined as C1C1 and Bw4/Bw4 individuals; the rest of the allotypes were considered unlicensed. However, since in this cohort it was not possible to restrict the analysis to AA haplotype subjects, the heterogeneous presence of activating KIRs in these subjects would be expected to modify the exact licensing status [60,61]. With this proviso, the key cytokine differences between the nominally licensed and unlicensed groups were concordant with the CD cohort: notably, elevated production in the licensed group of CCL-5, MIP-1β, IFN-γ, Granzyme B, and TNF-α (Figure 2-9); and, similar

overall features by cluster analysis (Figure 2-10). These findings indicate that KIR-mediated licensing results in a characteristic cytokine and chemokine program, regardless of the disease setting.

Licensed NK cells are polyfunctional in pro-inflammatory cytokine production at the single-cell level.

We wondered whether KIR licensing resulted in a mosaic of NK cells devoted to individual cytokines, or conversely, single cells expressing a polyfunctional cytokine program. To assess the cytokine secretion profile of individual NK cells, we utilized single cell barcode chips (SCBCs), [58] a high-throughput microfluidics platform. With SCBCs, single cells or a small number of cells are separated into thousands of microchambers on chip, where the production of 19 cytokines is simultaneously and independently measured during a 12-hour period. This technology recently has previously been extensively validated, and its utility in studying immune cell response has been demonstrated.[58] We compared single NK cell cytokine secretion between 2 C1/C1 Bw6/Bw6 and 2 $C2^+$ $Bw4^+$ CD patients. When we analyzed SCBC microchambers containing 1 to 4 cells, licensed NK cells exhibited a higher output of multiple cytokines, including TNF-α, MIP-1β, GM-CSF, IFN-γ, IL-2, IL-6, and CXCL-10 (see scatterplots and heatmap, Figure 2-11 and 2-12). PCA analysis of the single cell data from four CD patients with a licensing genotype showed that their NK cells contained a subset that produced effector proteins (CCL-5, TNF-α, IFN-γ, MIP-1β, and IL-6), which in contrast were barely detected in NK cells from patients without a licensing genotype (Figure 2-13). When we analyzed the co-expression of certain highly

expressed cytokines, namely TNF-β and GM-CSF, or IL-6 and MIP-1β, the prevalence of double-positive signals in multi-cell chambers was higher than the expected frequencies if the cells were acting independently (Figure 2-14). This suggests functional cross-talk between co-cultured NK cells, and deserves further study.

When we characterized the composition of cells producing 1, 2, 3, 4, 5, and >5 cytokines, we observed that a higher fraction (50-60% vs. 20-30%) of strongly licensed NK cells produced at least one cytokine, and half of them secreted 2 or more cytokines (Figure 2-15), supporting a polyfunctional phenotype of licensed NK cells. These findings combined demonstrated that licensed CD patient NK cells were reprogrammed for enhanced production of cytokines contributing to a chronic inflammatory state *in vivo*.

The NK cell subset expressing licensing KIRs contributes to the polyfunctional cytokine production of a licensed individual.

Within each individual, KIRs are stochastically expressed by NK cells, which result in a composite of licensed and unlicensed NK cells. [62] For example, in an AA haplotype healthy subject (who bears both KIR2DL3 and KIR3DL1 genes), the NK population includes cells that are single-positive, double-positive, or double-negative for these receptors. Accordingly, when such a subject is HLA-C1C1 (C1, ligand for KIR2DL3) and HLA-Bw6/Bw6 (Bw6, non-ligand for KIR3DL1), the $KIR2DL3^+$ NK cells are licensed, and the $KIR2DL3^-$ NK cells are unlicensed. We therefore tested the prediction that the licensing-associated polyfunctional cytokine production is a selective feature of the NK cell subset expressing licensing KIRs. We sorted licensed ($CD3^-$

$CD56^{dim}KIR2DL3^{+}KIR3DL1^{-}KIR2DL1^{-}$) and unlicensed ($CD3^{-}CD56^{dim}KIR2DL3^{-}$) subsets, achieving a high purity after sorting acquisition (>95%). These subpopulations were then evaluated for their single cell cytokine production capacity [58]. 77% of the $KIR2DL3^{+}$ NK cells co-expressed a large amount of CCL-5 and MIP-1b upon stimulation, whereas virtually no (1%) $KIR2DL3^{-}$ NK cells co-expressed these proteins. MIP-1β $^{+}$ $KIR2DL3^{+}$ NK cells also co-expressed IFN-γ, with a considerable fraction being IFN-γ/TNF-α double-producers, whereas $KIR2DL3^{-}$ NK cells were low for MIP-1β and silent for IFN-γ and TNF-α. Interestingly, more than 50% of MIP-1β $^{+}$ $KIR2DL3^{-}$ NK cells co-expressed TGF-β1, a regulatory cytokine rarely produced by MIP-1β $^{+}$ $KIR2DL3^{+}$ NK cells (Figure 2-16).

In $KIR2DL3^{-}$ NK cells, 65% were silent for all cytokines; 22% expressed one cytokine, and only 13% secreted 2 or more cytokines. In sharp contrast, 99% of $KIR2DL3^{+}$ NK cells secreted at least one cytokine. Among them, 15% expressed one (CCL-5 or MIP-1β); one third expressed two cytokines (typically $CCL\text{-}5^{+}MIP\text{-}1\beta^{+}$), and another one third expressed three cytokines (mostly IFN-γ $^{+}$MIP-1β $^{+}$$CCL\text{-}5^{+}$); and, a substantial fraction (10%) produced four cytokines. Due to the high purity of the sorted subpopulations, these findings could not be attributed to an artifact of contaminating cells, but instead reflect the intrinsic functional scope and heterogeneity within each of these NK cell subpopulations. Accordingly, they indicate that licensed NK cells were able to produce a wide variety of cytokines, and were more polyfunctional than unlicensed NK cells (Figure 2-18). The reason for the functional heterogeneity observed in each subpopulation is uncertain, but is likely to reflect action of other cytokine and

activating receptor interactions that contribute to maturational and educational plasticity of the NK population [63,64].

Analysis of ~1,500 KIR2DL3^{+} and KIR2DL3^{-} single NK cells primarily resolved into two clusters: one cluster primarily contains KIR2DL3^{+} NK cells polarized towards a pro-inflammatory state (left cluster in Figure 2-17), and the second cluster primarily contains KIR2DL3^{-} NK cells polarized towards a more regulatory state, (right cluster of Figure 2-17, licensed and unlicensed NK cells are also indicated by the top red-blue row). Principal component analysis (PCA) showed that the two subsets have strikingly opposing characteristics, particularly distinguished in their production of effector proteins (such as TNF-α, IFN-γ, and chemokines) shown in the second component (19% of variance, Figure 2-19). It is important to emphasize that KIR2DL3^{-} NK cells were not cytokine silent, but instead polarized for a distinct program of cytokine production, including IL-12, TGF-β, and TNF-β. Taken together, our findings indicated that licensing mediated by KIR2DL3/HLA-C1 interaction conferred pro-inflammatory immune mediator production program in NK cells.

IFN-γ, TNF-α, and IL-6 are accountable 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 co-cultures (Figure 2-20 and 2-21). Neutralization of TNF-α alone had a great impact on CD4^{+} T cell proliferation, and this effect was specific compared to IgG1κ 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 20ng mL^{-1}, comparable to that produced by licensed NK cells (Figure 2-22). 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.

Supernatant of NK cells from genetically licensed individuals potently promoted T_H17 differentiation.

T helper 17 (T_H17) cells are crucial drivers for multiple chronic inflammatory diseases, including CD, [65,66] but there is little information about if or how NK cells might affect T_H17 formation or activity. The foregoing results indicated that licensed NK cells are robust producers of several cytokines, notably IL-6, important in T_H17 differentiation. [65,67,68] We therefore tested the effect of licensed NK cell cytokines on T_H17 differentiation. While the conditions for murine T_H17 differentiation have been well defined, a general agreement on the factors required for human T_H17 cell differentiation remains to be established. [65,67,68] In pilot studies, we first established that IL-17A and IL-22 producing T_H17 cells can be successfully induced from total peripheral CD4+ T cells

using a variety of cytokine combinations. The most effective were IL-23/IL-6/IL-1β/IL-2, IL-23/IL-6/IL-1β, and IL-6/IL-1β/IL-2 (Figure 2-23). To determine if licensed NK supernatant could promote T_H17 differentiation in total $CD4^+$ T cells, we titrated NK supernatants from a licensed CD patient (AA KIR and C1C1 HLA) into $CD4^+$ T cell cultures for 6-7 days, further propagated the cultures for another 6-7 days, and then assessed for IL-17A and IL-22 producing cells by flow cytometry. Compared to $CD4^+$ T cells polarized with IL-1β or IL-23 alone, addition of 50% licensed NK cell supernatant dramatically increased the percentage of $IL\text{-}17A^+$, $IL\text{-}22^+$, and $IL\text{-}17A^+IL\text{-}22^+$ cells (Figure 2-24 and Figure 2-25). The same effects were also observed with licensed NK supernatants alone, or in combination with both IL-1β and IL-23 (Figure 2-26).

Using the IL-23 plus NK supernatant condition, we analyzed NK cell supernatants from four different healthy subjects with a licensing genotype (AA KIR, C1C1 HLA). All of them showed T_H17 differentiation capacity, assessed from their higher percentages of $IL\text{-}17A^+$, $IL\text{-}22^+$, and $IL\text{-}17A^+IL\text{-}22^+$ cells (Figure 2-27). These findings consistently demonstrated that licensed NK cells secret immune mediators that can strongly promote T_H17 differentiation, either alone or synergistically with IL-23 and IL-1β. They also demonstrate that comparable T_H17-inducing cytokines are produced by licensed NK cells from healthy individuals.

IBD patients with licensing genotypes are more likely to respond to thiopurine therapy

To determine whether KIR-HLA genetics is correlated with thiopurine responsiveness, we used an IBD pediatric cohort of 134 with thiopurine therapy

response outcomes (Table 2-6). The primary end points for thiopurine response rate was determined by steroid-free remission at week 26.[69] We grouped the patients into licensed group (homozygous HLA-C1 or HLA-Bw4) and unlicensed group (all other allotypes), and compared their response rates. There is no significant difference observed between the two groups in total patients (p=0.287 by Fisher's exact test), but when we compared the two groups within the AA haplotype, there is a striking difference. 62.5% patients with licensing genotype went into remission at week 26, while only 20.0% patients with unlicensing genotype did (Table 2-7. Odds Ratio = 3.125, p=0.016 by Fisher's exact test). We also restricted our analysis to the AA haplotype CD patient subset, and the trend of difference between the two groups is observable, but due to the smaller sample size (30 CD instead of 39 IBD), the significance is reduced (p=0.056 by Fisher's exact test).

Discussion

The genetic presence of strong NK licensing KIR/ligand pairs (KIR2DL3/HLA-C1 or KIR3DL1/HLA-Bw4) affects several important chronic inflammatory diseases: elevated susceptibility to CD, Celiac disease, spondyloarthropathy, psoriatic arthritis; enhanced resolution of Hepatitis C virus (HCV) infection, and slower progression in HIV-1 infection.[70-76] Understanding the nature of KIR contribution to disease susceptibility or protection is crucial for developing diagnostic and treatment strategies (Figure 2-28). However, biologic study of KIR-mediated disease association has been challenging due to two levels of complexity. First, it is difficult to dissect the conflicting roles of KIRs in NK cell licensing and in target-induced inhibitory signaling. In this study, we have studied NK cell function independent of target cell interaction, to exclude the role of KIR-mediated inhibitory signaling and thereby focus solely on KIR-mediated NK licensing. Second, polymorphic composition and functions of KIR haplotypes, and the independent assortment of their cognate HLA class I ligands, confound the biologic study of NK licensing by individual KIRs. By focusing on the simplified AA haplotype, 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 T_H17 differentiation, which provide a mechanistic basis for their genetic association to 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 was preserved in two independent patient cohorts, CD and MS; and functionally, licensed NK cell

cytokines from both CD and healthy subjects comparably augmented $CD4^+$ T cell polarization. While the licensing-associated cytokine program was preserved among all cohorts, it is notable that there were significant differences in the detailed NK cell cytokine production in the CD and MS cohorts: GM-CSF, perforin, and IL-15 in the MS cohort; IL-6, IL-4, and TNF-β in the CD cohort. These differences may be due to the impact of non-A haplotype KIR genes included in the MS cohort; [60,61] the larger size of the MS cohort; and, disease- or treatment-related effects on the NK cell population.

A striking finding of this study was the selective capacity of licensed NK cell cytokines to efficiently drive IL-17A and IL-22 production, as well as populations co-expressing IL-17A and IL-22. This is in part attributable to IL-6, a cytokine produced by licensed NK cells known to be important in T_H17 polarization. However, other NK-produced cytokines may be relevant, since in several culture conditions, T_H17 polarization by NK cell supernatants exceeded that expected for IL-6 alone. Through IL-6 (and perhaps other cytokines), licensed NK cells synergize with IL-23 and IL-1b to facilitate T_H17 differentiation, indicating that licensed NK cell secretions can collaborate with other cells in the local tissue compartment (dendritic cells or macrophages) to promote a more pro-inflammatory environment shaping $CD4^+$ or $CD8^+$ T cell responses. [77,78]

The fundamental mechanism by which NK licensing yields a distinct NK cell cytokine program is still emerging. Recent work indicates that upon NK licensing, activating NK cell receptors become dynamically compartmentalized in membrane nanodomain that permits full signaling reactivity. [79] In mice and humans, licensed NK cells gain higher sensitivity to cytokine stimulation,[24,27,73] but how the intracellular

signaling network is rewired after NK licensing is unknown. Our inter- and intra-individual analysis of KIR-mediated licensing indicates that this rewiring affects a remarkably broad range of cytokines, which poses further challenges and conditions to the underlying mechanism of NK licensing. The exceptional breadth and pro-inflammatory cytokine profile of licensed NK cell is an important finding of this study, as well as evidence that this cytokine production lowers the threshold for $CD4^+$ T cell activation. It is also conceivable that licensed NK cells, through their robust cytokine production, may contribute to the initiation of the pro-inflammatory process prior to the innate or immune activation of dendritic cells and macrophages. [27,40]

The most significant finding in this study is that thiopurine responsiveness in IBD patients can be predicted based on their KIR-HLA genetics. And this finding can be potentially used to guide the clinical use of thiopurine agents. It is worth noting that the significant difference between licensing versus unlicensing genotype are restricted to individuals with homozygous A haplotype (Table 2), suggesting the presence of other KIR genes in B haplotype individuals may complicate the prediction for their thiopurine responsiveness.

Finally, as the pathogenesis of Crohn's Disease is ultimately driven by intestinal lymphocytes; it is desirable to confirm these properties of licensed NK cells resident in the gastrointestinal tract. However, there are substantial technical difficulties in obtaining useful numbers of intraepithelial (IE) or lamina propria (LP) NK cells for the required functional studies. Since the analysis requires patients with informative combinatorial KIR and HLA genotypes, useful colonic resection specimens are uncommon, and require staging by substantial genotyping prior to resection.

Identification of patients from genotyped cohorts undergoing colonscopic procedures is an appealing approach. However, the abundance of $CD3^{-}CD56^{+}$ NK cells in IEL and LP are ~18.5% and ~10%, respectively.[80,81] Therefore, in order to harvest 200,000 mucosal NK cells, we would need in >1-2 million lymphocytes, hence ~20 biopsy 2 mm^2 from each patient[82], which is outside the scale of approved research biopsy sampling. Therefore, while the evaluation of mucosal NK cells is appealing, it is outside the scope of the present study.

In summary, our finding of the pro-inflammatory role of licensed NK cells on adaptive immunity is mechanistically important, as it offers a fresh biologic diagram accounting for the impact of KIR-HLA genetics on IBD and other chronic inflammatory diseases. Furthermore, our finding is clinically beneficially, as the KIR-HLA genetics can be used to stratify IBD patients for thiopurine therapy.

Figures and Tables

Figure 2-1

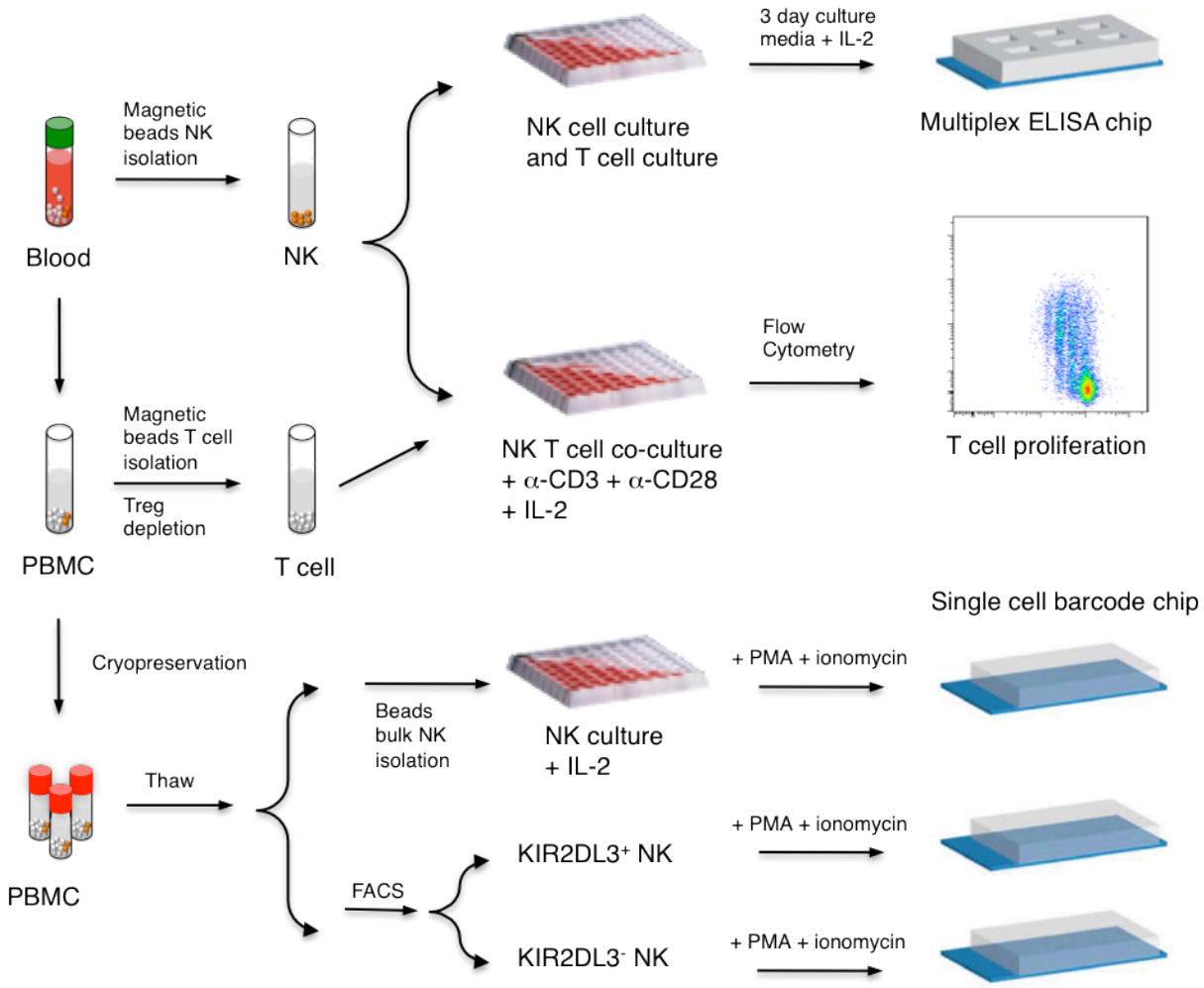

Figure 2-1. Diagram of CD patient blood sample processing pipeline and immune monitoring technologies utilized.

Whole blood samples were used for direct isolation of CD56+ NK cells and PBMC, followed by T cell isolation with depletion of T regulatory (Treg) cells. NK cells and Treg-depleted T cells were put in co-cultured or separately, with 2ng mL^{-1} (26 I.U.) IL-2 and antigenic stimulation for T cells as indicated. $CD4^+$ T cell proliferation was subsequently analyzed by flow cytometry. NK and T cell supernatant were analyzed using multiplex

ELISA chip within one batch. Aliquots of PBMC were cryo-preserved, and thawed for SCBC analysis with or without pre-sorting.

Figure 2-2

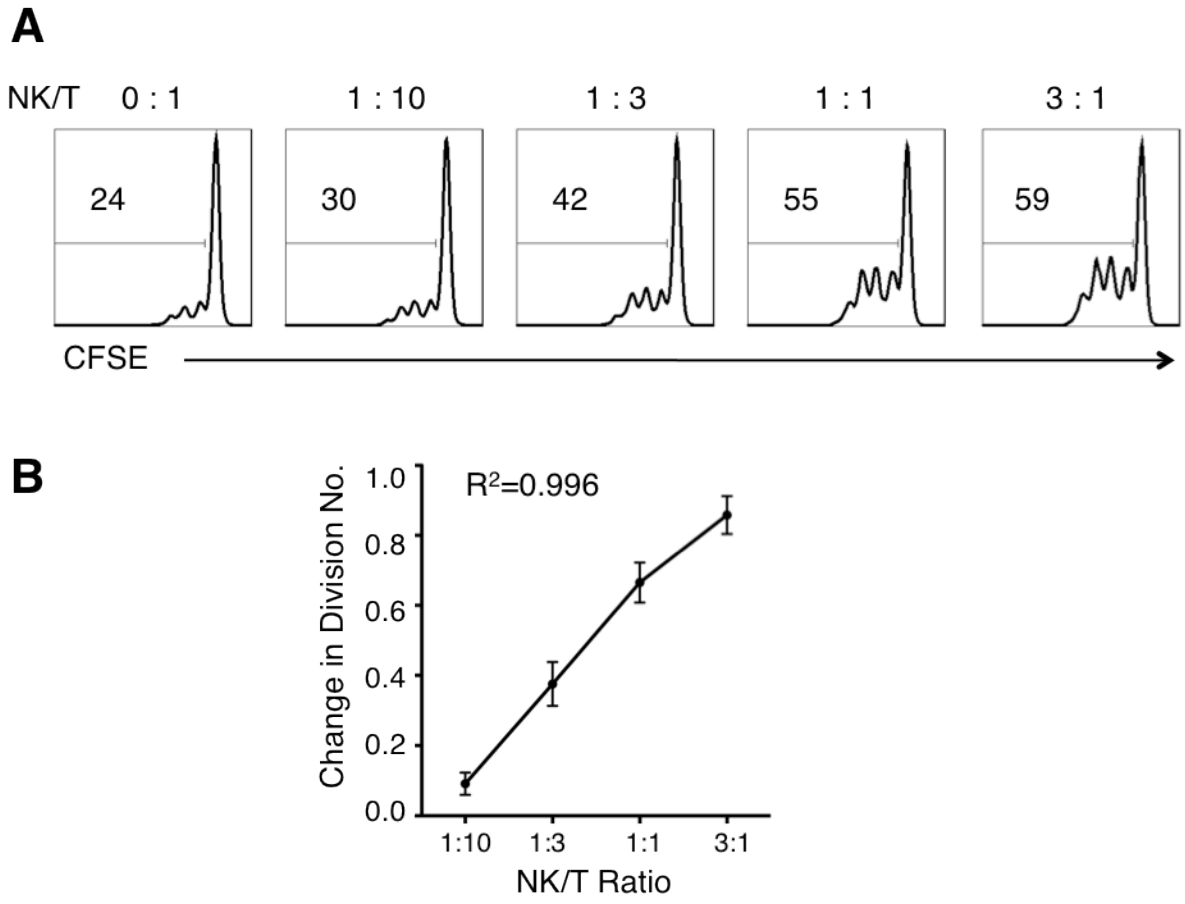

Figure 2-2. Licensed NK cells promote autologous $CD4^+$ T cell proliferation.

NK cells and autologous T cells were isolated from AA haplotype CD patient peripheral blood, stimulated with 1.5μg mL^{-1} immobilized anti-CD3 anti-CD28, and co-cultured in 2ng mL^{-1} (26 I.U) IL-2 for three days. (A) Histograms of $CD4^+$ T cell CFSE dilution after co-culturing with NK cells at the NK/T ratios as indicated, for a representative C1C1 CD patient. 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 co-culture/mean CFSE intensity of T cell alone.

Figure 2-3

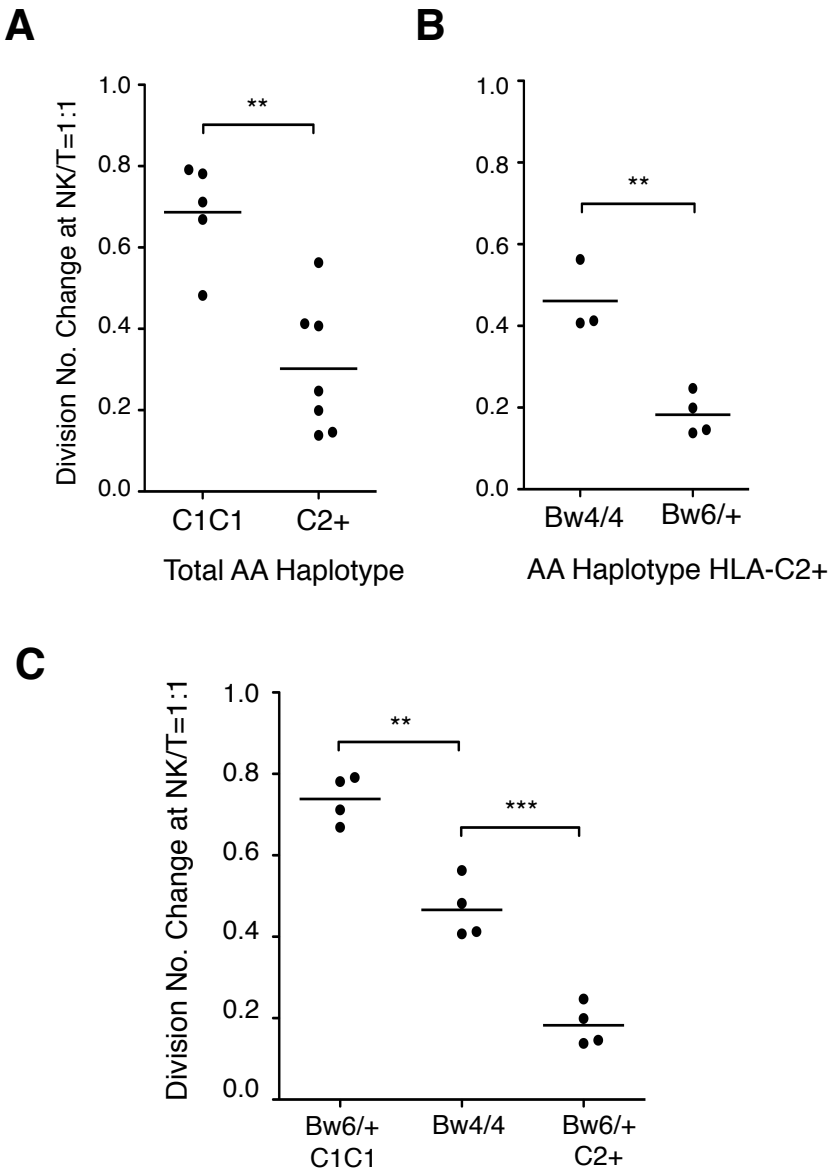

Figure 2-3. The capability of NK cells of promoting CD4$^+$ T cell proliferation is directly correlated with their strength of KIR licensing.

Comparison of change in CD4$^+$ T cells division number at NK/T = 1:1, between (A) AA haplotype C1C1 and C2$^+$ patients, (B) in AA haplotype C2$^+$ patients between Bw4/4 and Bw6/$^+$, and (C) AA haplotype patients C1C1Bw6/$^+$, Bw4/Bw4, and C2$^+$Bw6/$^+$. (n = 3 to 4 , Student's t-test, two-tailed. ** $p < 0.005$; *** $p < 0.0005$).

Figure 2-4

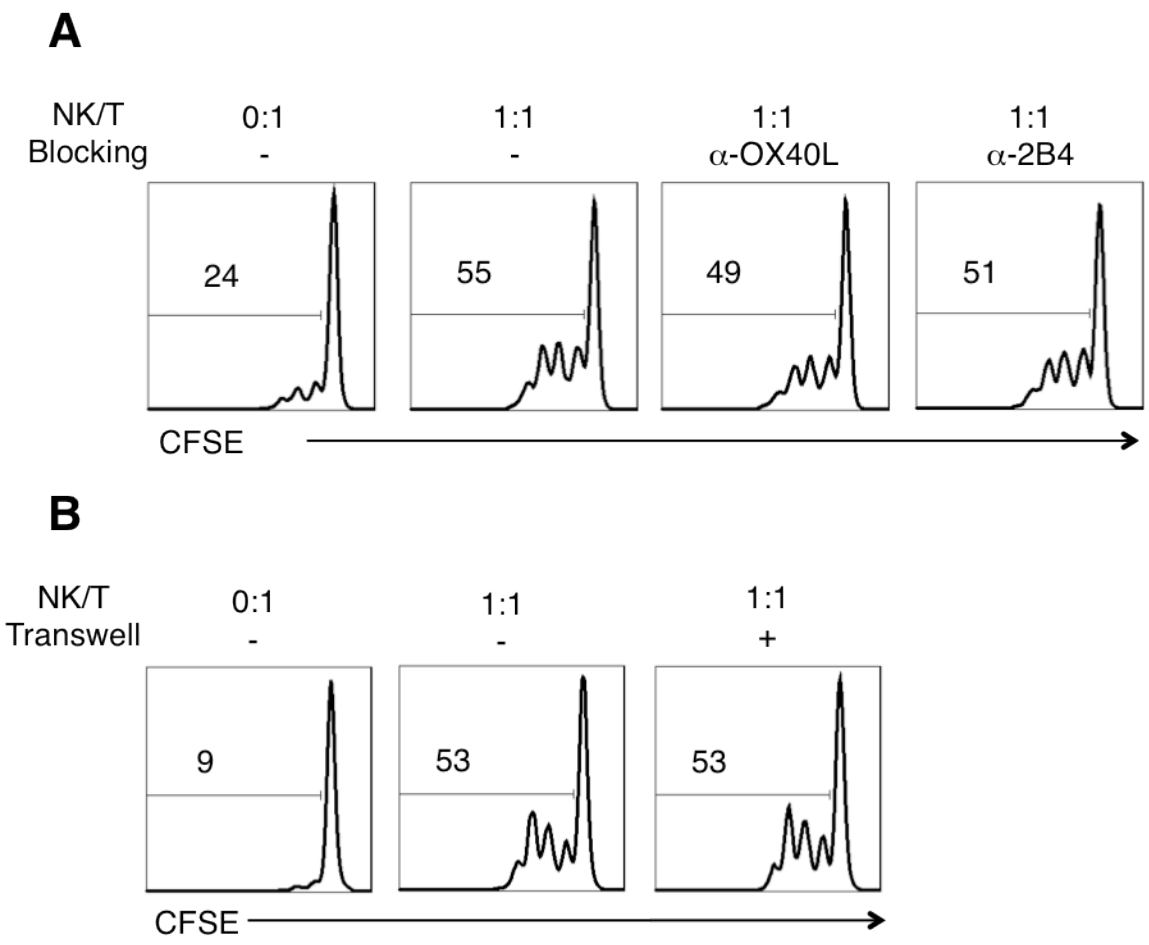

Figure 2-4. The interaction between licensed NK cells and $CD4^+$ T cells is contact-independent.

(A) Histograms of $CD4^+$ T cell CFSE dilution in the absence of (left two) or in the presence (right two) of the indicated blocking antibodies at 10μg mL^{-1}. (B) Histograms of $CD4^+$ T cell CFSE dilution at the indicated NK/T ratio without physical separation of NK cells and T cells (left two) or with separation by 1.0 μm pore size transwells (right one). The numbers in each histogram indicates the percentage of cells proliferated.

Figure 2-5

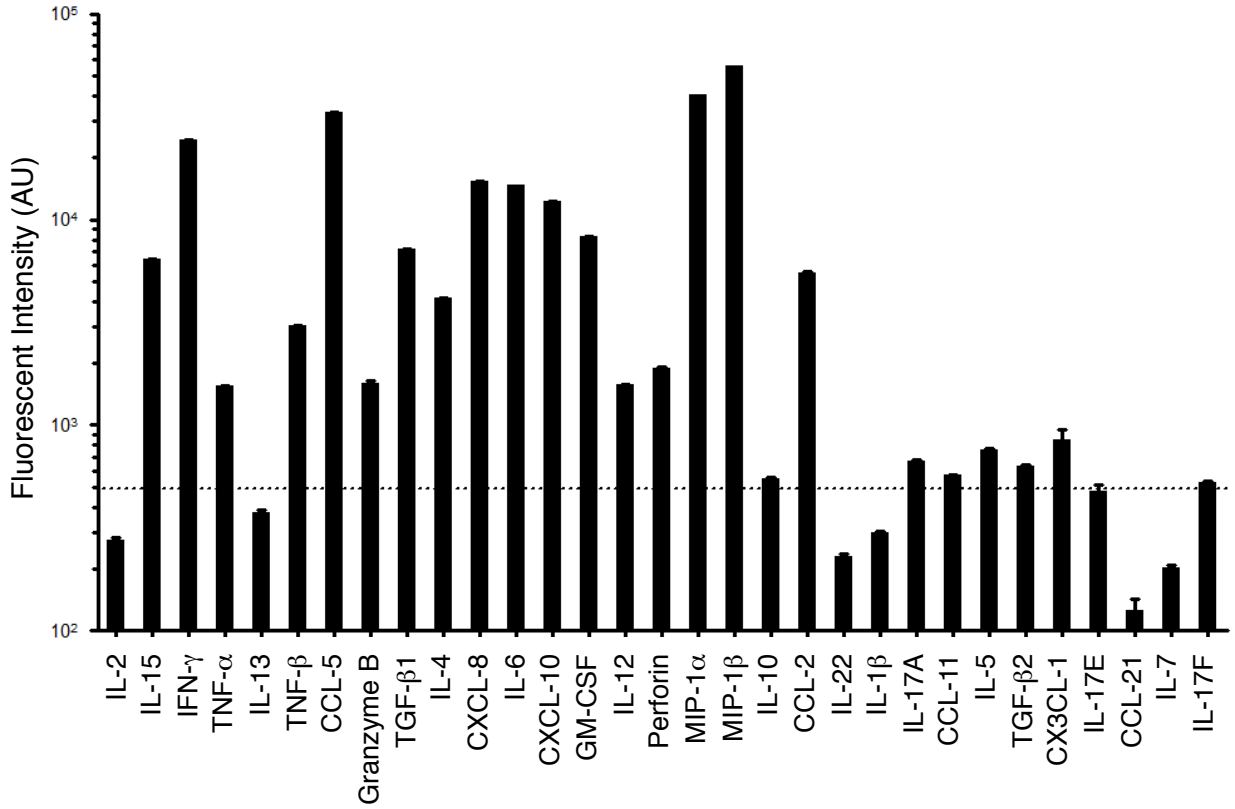

Figure 2-5. Screening for high abundant immune mediators secreted by NK cells using multiplex ELISA.

Barplot of florescence intensity (Mean ± s.e.m, A.U. arbitrary unit) of an initial panel of 31 protein markers from 72-hour NK culture media, n = 8. The dash line shows the detection threshold at 500 A.U.

Figure 2-6

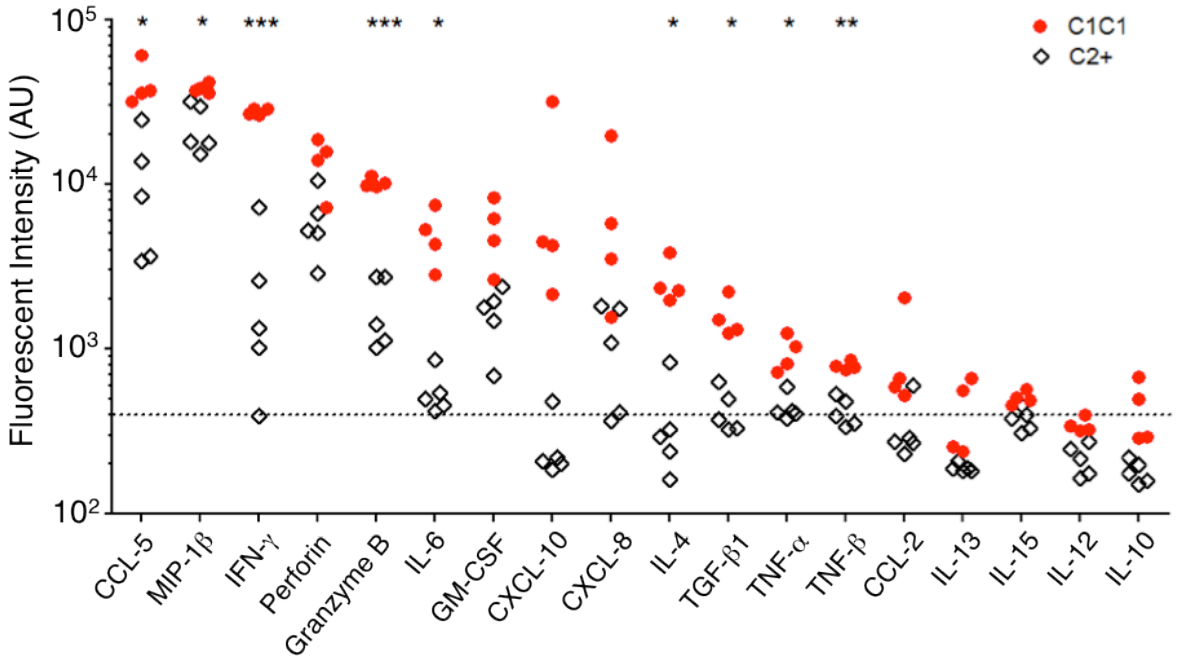

Figure 2-6. AA haplotype C1C1 CD patient NK cells have distinct cytokine production levels compared to $C2^+$ CD patient.

(A) Univariate comparison of cytokine production level of bulk culture NK cells from licensed (C1C1, solid dot) and unlicensed ($C2^+$, open Square) CD patients. The vertical axis shows the fluorescence intensity. (n = 4 to 5, P values are calculated using two tailed Student's t-test, adjusted for multiple comparison by FDR, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$). The dash-line indicates the detection threshold. Secretion profiles were measured by multiplex ELISA.

Figure 2-7

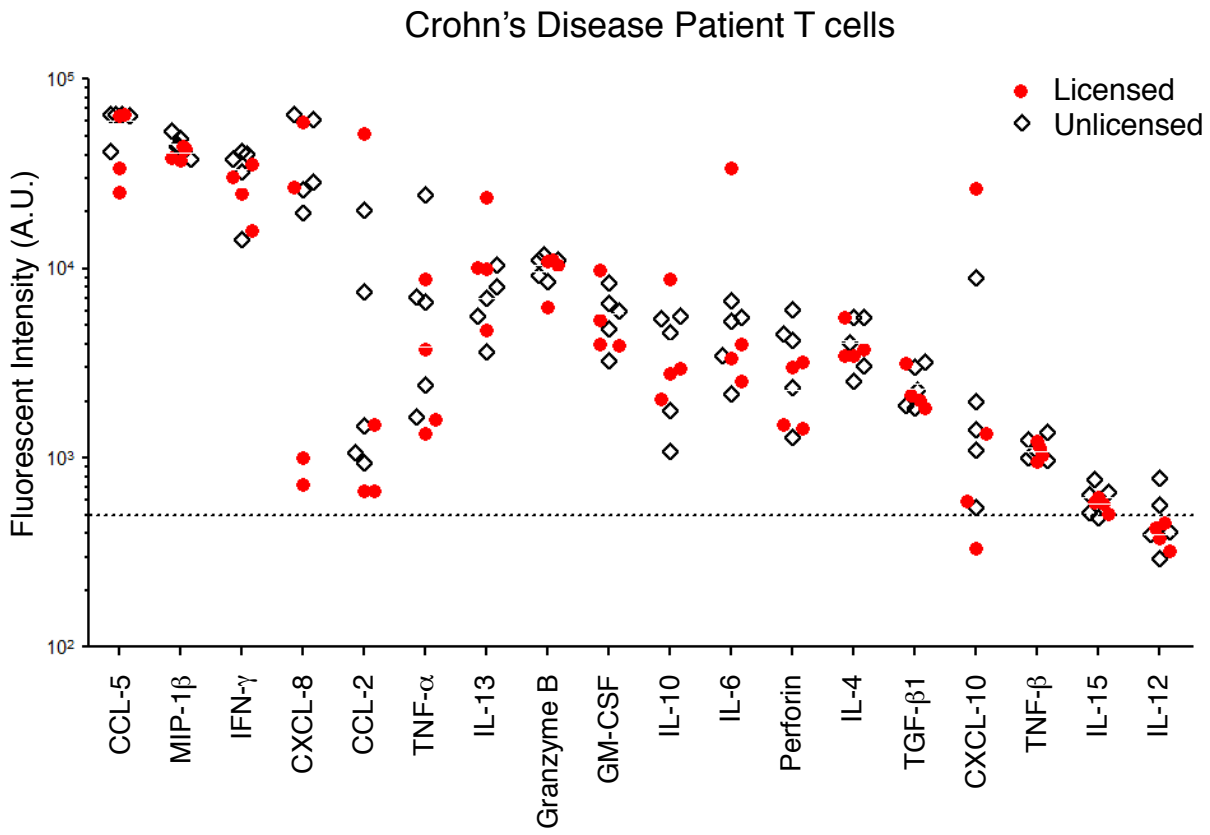

Figure 2-7. AA haplotype C1C1 CD patient T cells have similar cytokine production levels as $C2^+$ patient.

T cells were stimulated with 1.5 μg mL^{-1} immobilized anti-CD3/CD28 and 2 ng ml^{-1} IL-2 for three days. Y axis shows the fluorescence intensity of each cytokine from T cell supernatant. n = 4 to 5. Solid dot: C1C1, Open Square: $C2^+$. The dash line shows the detection threshold at 500 AU.

Figure 2-8

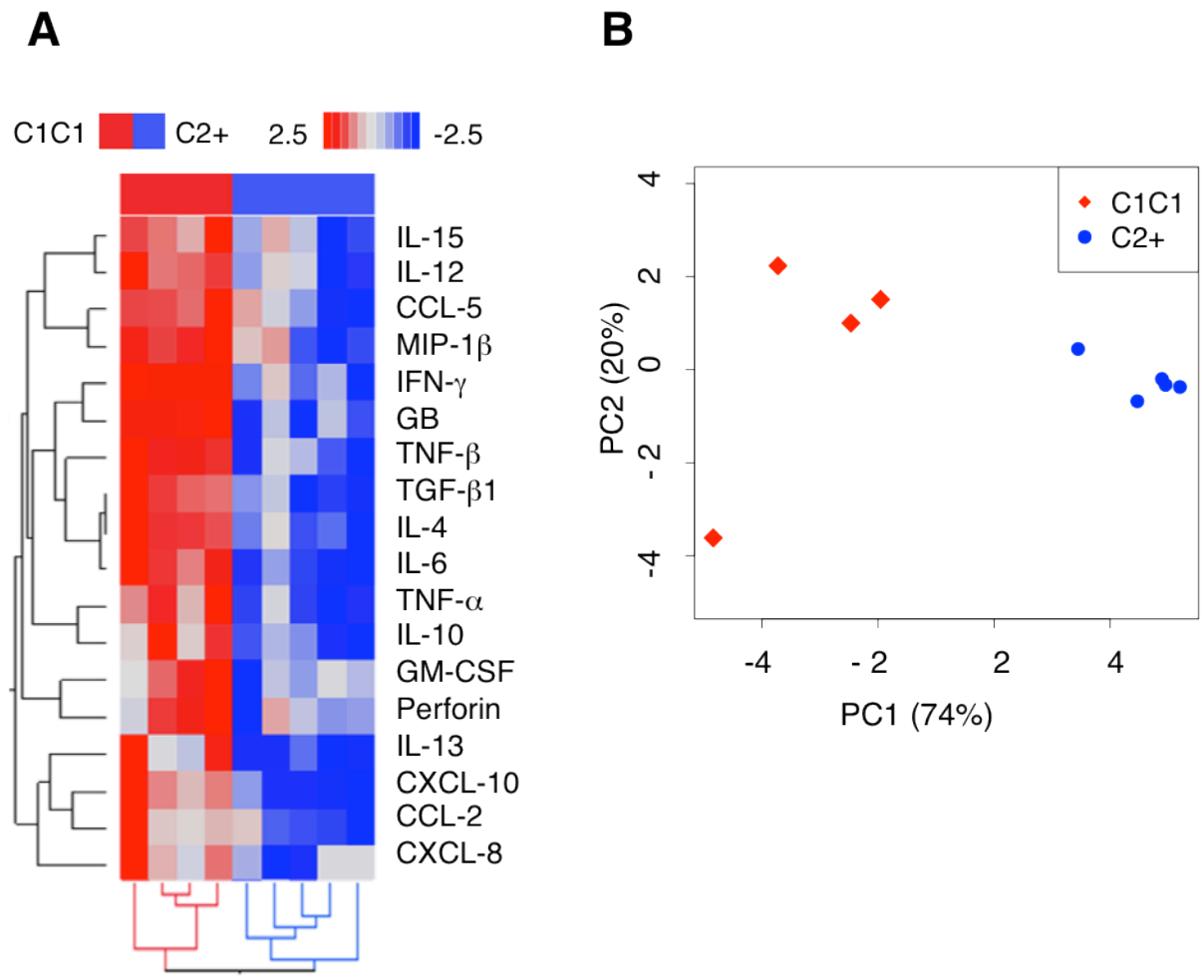

Figure 2-8. NK cells from AA haplotype C1C1 patients have distinct cytokine secretion patterns compared to those from C2$^+$ patients.

(A) Hierarchical clustering of the bulk cytokine production profile of NK cells from C1C1 (licensend, red) and C2$^+$ (unlicensed, blue) CD patients. Each row represents one protein indicated on the right, and each column represents one patient. (n= 4 to 5). (B) Principal Component Analysis of normalized C1C1 and C2$^+$ NK 72-hour culture supernatant secretion profiles, represented by the first two components. The

percentages of variation explained by each component were shown in parentheses. n= 4 to 5, C1C1 (red square), $C2^+$ (blue dot).

Figure 2-9

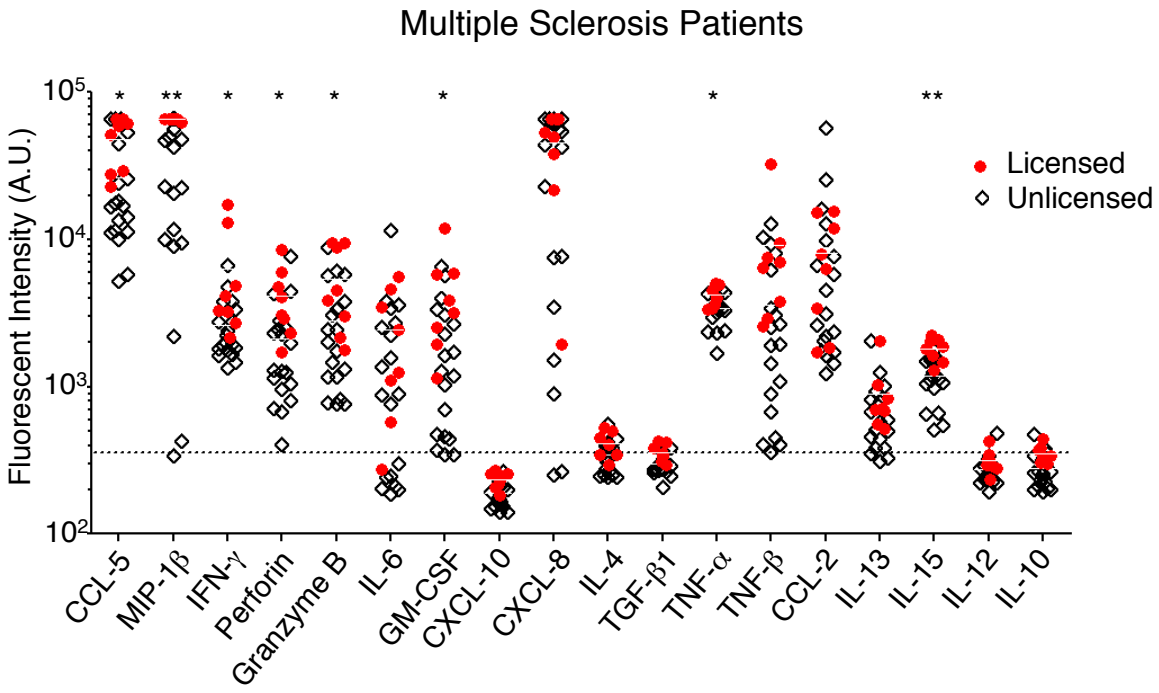

Figure 2-9. C1C1 MS patient NK cells have distinct cytokine production levels compared to $C2^+$ MS patient.

Univariate comparison of cytokine production from licensed (all C1C1 and all Bw4/Bw4, red solid dot) and unlicensed (all other genotype, open square) MS patients. (n = 8 to 19, P values are calculated using two tailed Student's t-test, adjusted for multiple comparison by FDR, * $p < 0.05$; ** $p < 0.005$). The dash line shows the detection threshold.

Figure 2-10

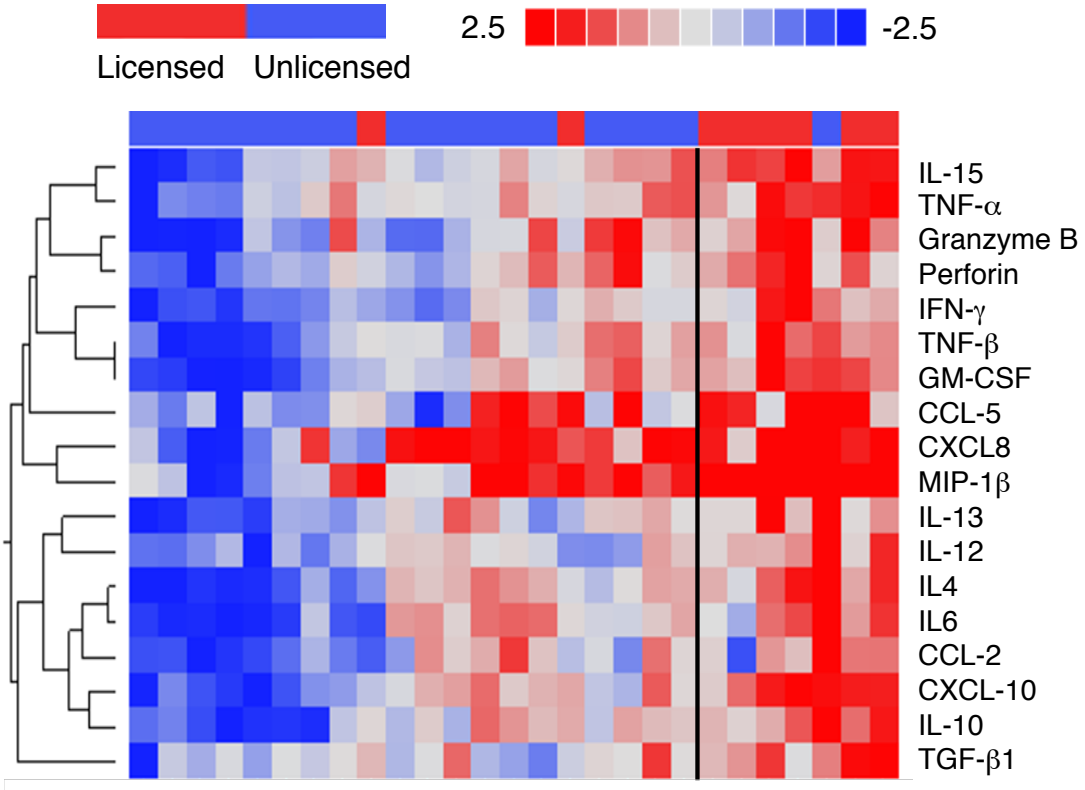

Figure 2-10. Heatmap analysis of MS patient licensed and unlicensed NK cell secretion profiles.

Clustering of normalized licensed and unlicensed NK cell 72-hour culture (in 2ng ml^{-1} IL-2) supernatant secretion profiles. Horizontal color bars on top of the heatmap represents licensed (red bar) and unlicensed (blue bar) patients. Each row represents on cytokine, each column represents one patient. n= 8 to 19.

Figure 2-11

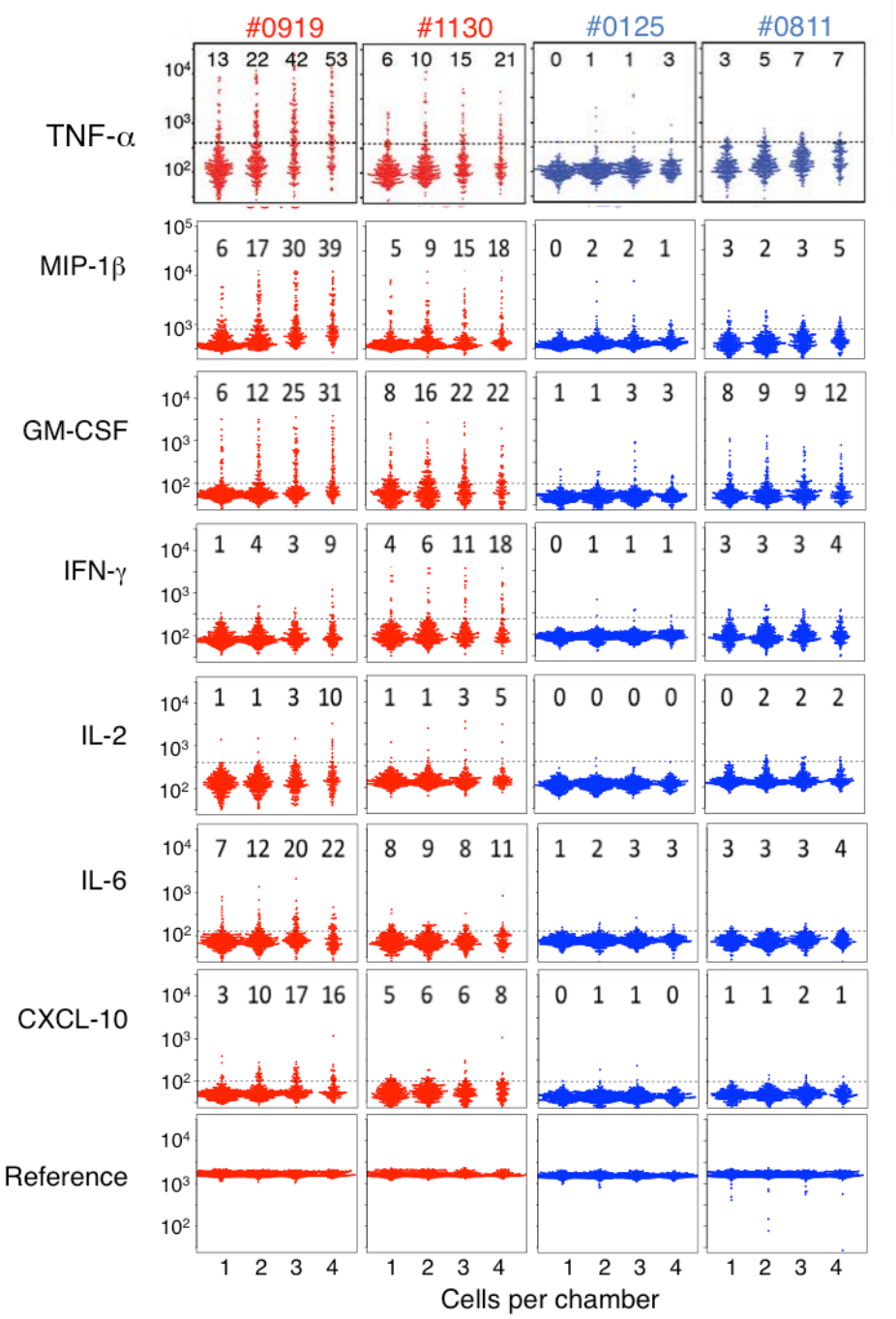
#0919
#1130
#0125
#0811
TNF-α
MIP-1β
GM-CSF
IFN-γ
IL-2
IL-6
CXCL-10
Reference
13 22 42 53
6 10 15 21
0 1 1 3
3 5 7 7
6 17 30 39
5 9 15 18
0 2 2 1
3 2 3 5
6 12 25 31
8 16 22 22
1 1 3 3
8 9 9 12
1 4 3 9
4 6 11 18
0 1 1 1
3 3 3 4
1 1 3 10
1 1 3 5
0 0 0 0
0 2 2 2
7 12 20 22
8 9 8 11
1 2 3 3
3 3 3 4
3 10 17 16
5 6 6 8
0 1 1 0
1 1 2 1
1 2 3 4
Cells per chamber

Figure 2-11. Univariate comparison of secretions between C1C1 and $C2^+$ CD patient NK cells using SCBC.

MIP-1β, GM-CSF, IFN-γ, IL-2, IL-6, CXCL-10, and reference signals from 1-4 cell SCBC microchambers from C1C1 (#0919, #1130, red) and $C2^+$ (#0125, #811, blue) CD patients.

Figure 2-12

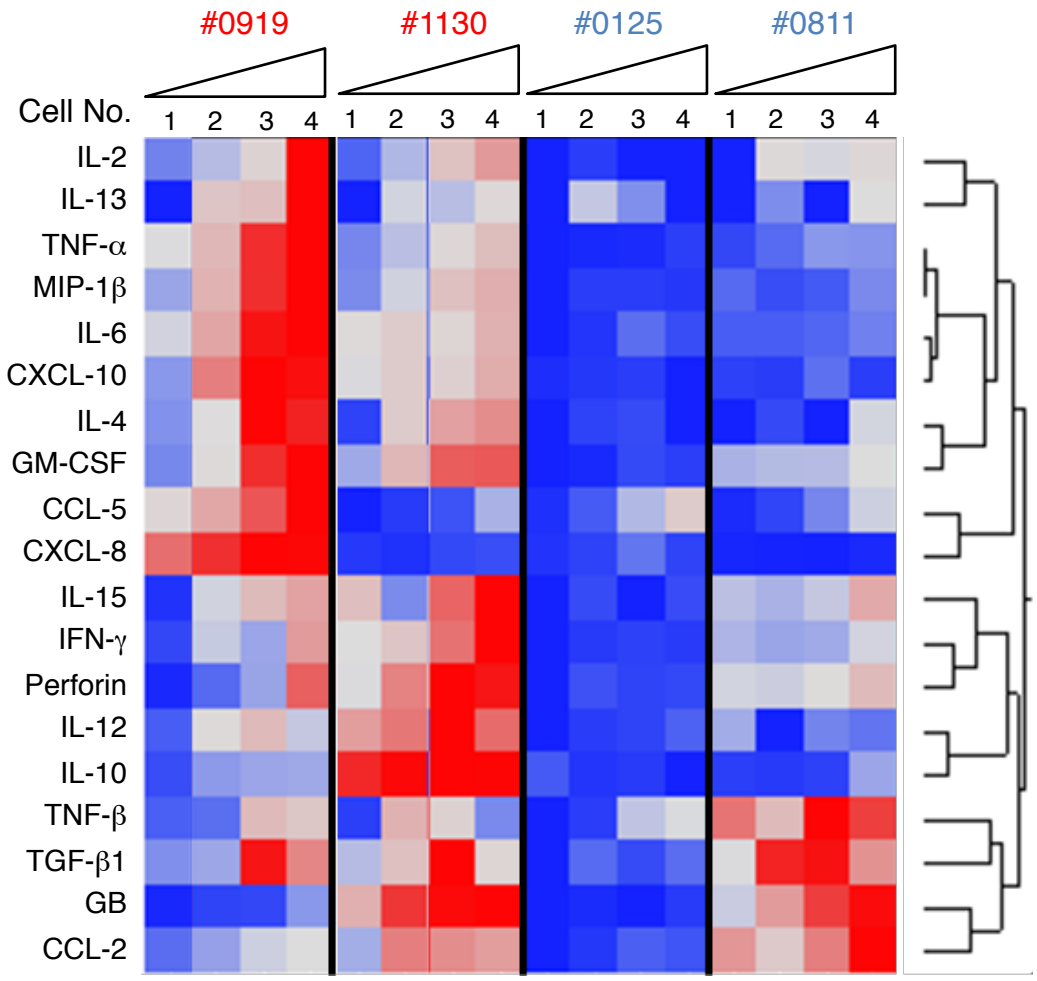

Figure 2-12. Heatmap analysis of MS patient licensed and unlicensed NK cell secretion profiles.

Clustering of normalized licensed and unlicensed NK cell 72-hour culture in 2ng ml^{-1} IL-2 supernatant secretion profiles. Horizontal color bars on top of the heatmap represents licensed (red bar) and unlicensed (blue bar) patients. Each row represents on cytokine, each column represents one patient. n= 8 to 19.

Figure 2-13

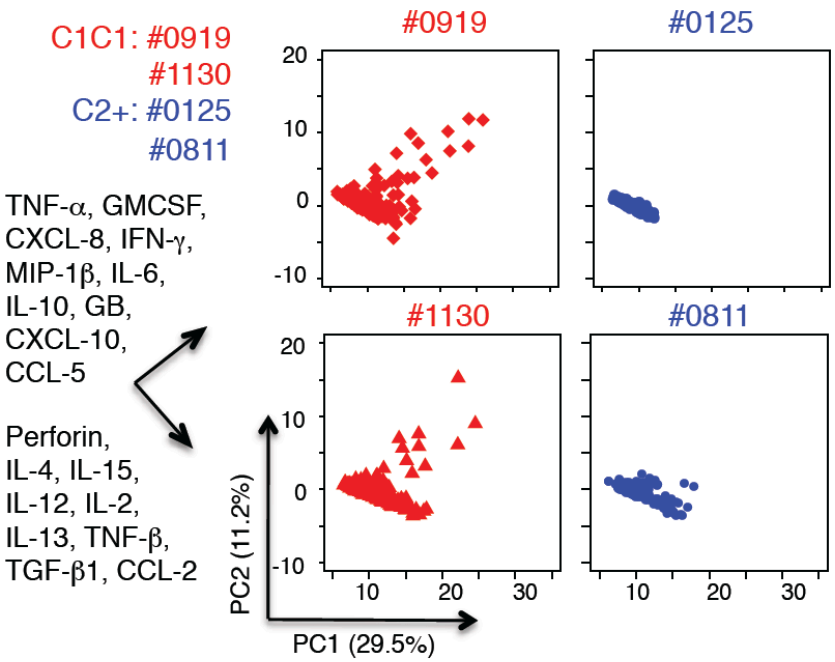

Figure 2-13. NK cells from AA haplotype HLA-C1C1 CD patients produce multiple effector immune mediators that are lacked from HLA-C2^{+} CD patients.

PCA of single NK cell measurements from the four CD patients. 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 plot.

Figure 2-14

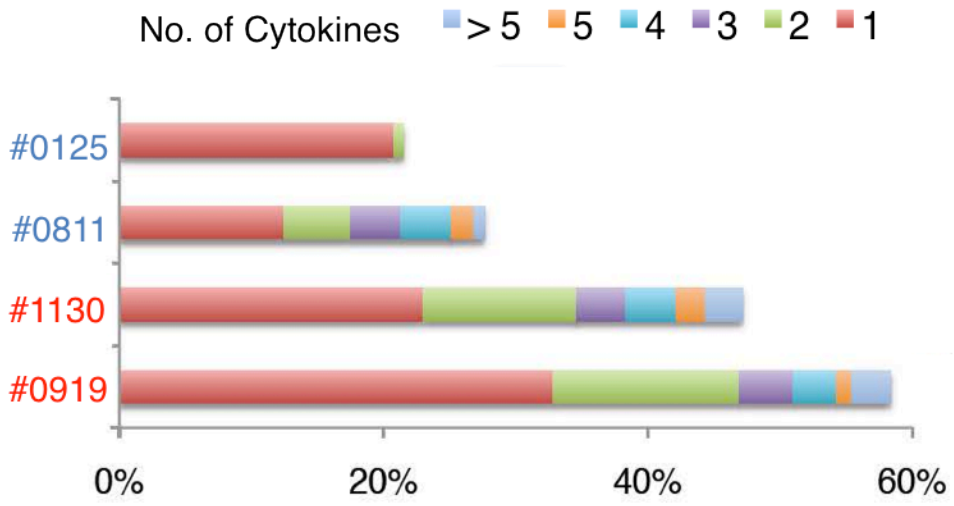

Figure 2-14. At single cell level, NK cells from HLA-C1C1 CD are more polyfunctional than those from HLA-C2^{+} CD patients.

Bar graph of NK cell polyfunctionality defined by the number of cytokines each NK cell secrets. The percentage of single NK cells producing 1, 2, 3, 4, 5, and > 5 cytokines for the four patients studied is showed by a different color.

Figure 2-15

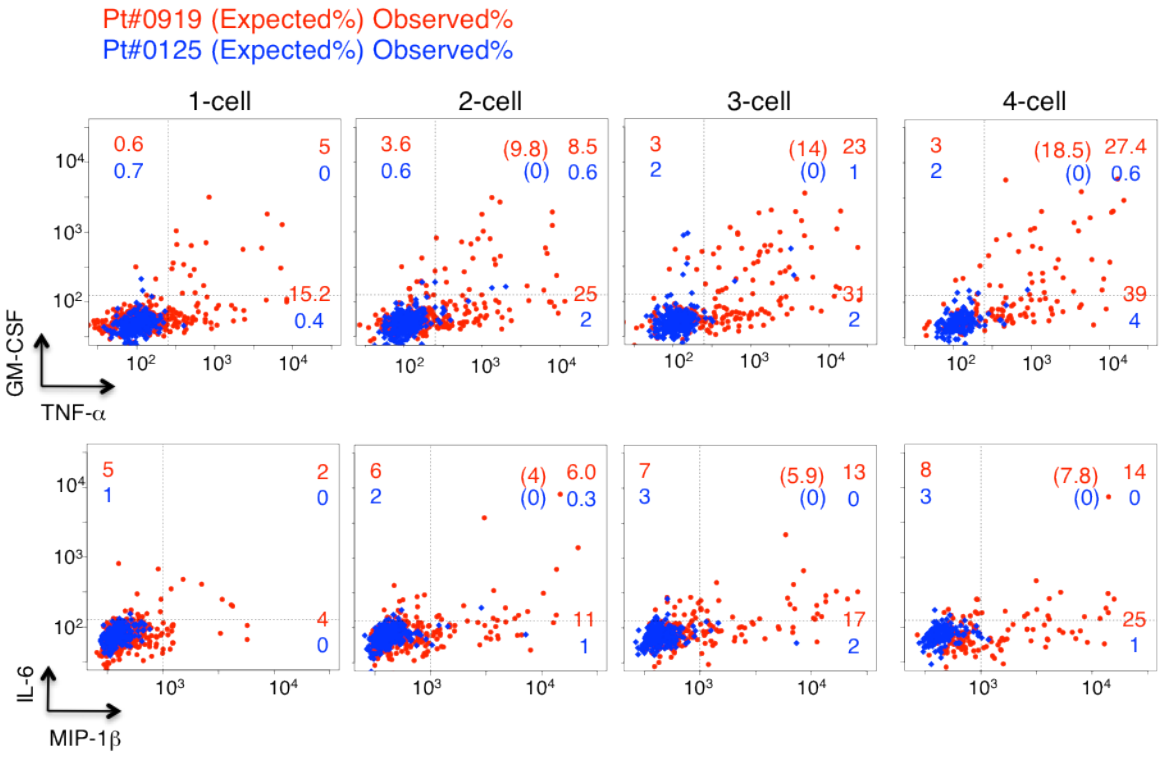

Figure 2-15. Comparison of secretion profiles between C1C1 and C2^{+} CD patient NK cells.

2-D scatter plot of cytokine signals of licensed (#0919, red) and unlicensed (#0125, blue) patient NK cells from SCBC 1-4 cell microchamber. Numbers in each quadrant indicated the percentage of signals in that quadrant. Numbers in parentheses indicated the expected frequencies of signals if cells were acting independently, calculated from 1-cell signals.

Figure 2-16

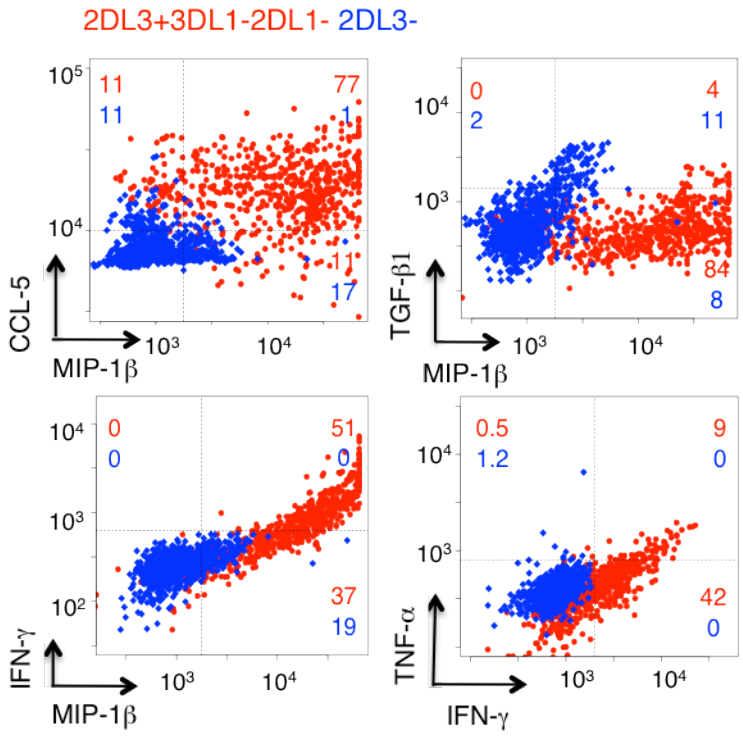

Figure 2-16. Licensed and unlicensed NK cell subsets have differentially polarized cytokine capacity.

Peripheral blood NK cells from an AA haplotype C1C1 healthy donor were sorted for KIR2DL3^{+}KIR3DL1^{-} KIR2DL1^{-} (licensed) and KIR2DL3^{-} (unlicensed) NK subsets, stimulated with PMA/ION for 12 hours, and analyzed using SCBC. 2-D scatter plot of representative cytokine production levels from licensed (red) and unlicensed (blue) NK cell subsets. Axis units are fluorescence intensity. A gate is determined for each cytokine. The number in each quadrant represents the percentage of cells in that quadrant.

Figure 2-17

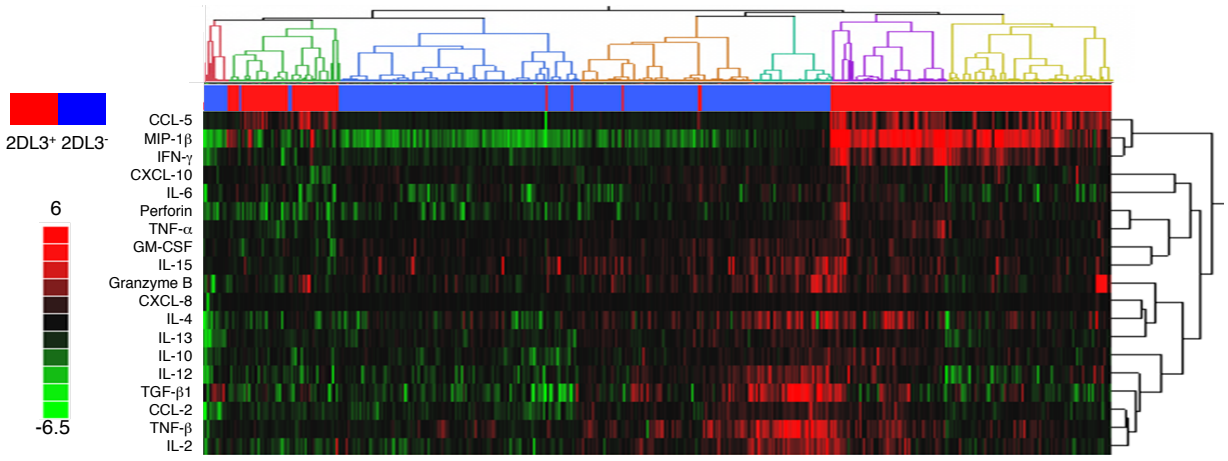

Figure 2-17. Hierarchical clustering of cytokine measurements from licensed (red) and unlicensed (blue) NK cells at the single cell level.

Each column represents one single cell, and each row presents one cytokine. The color scale shows the difference in standard deviation.

Figure 2-18

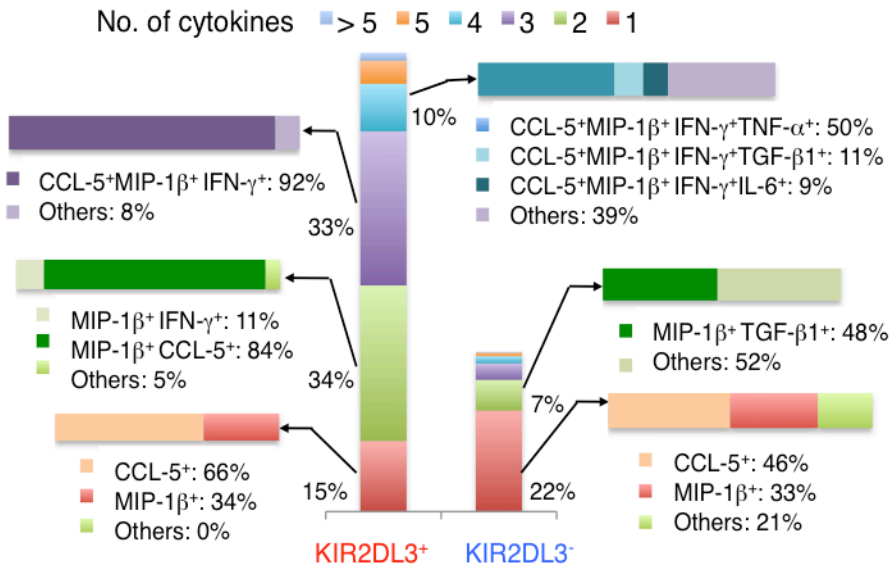

Figure 2-18. Licensed NK cells from an AA haplotype individual are more polyfunctional compared to unlicensed NK cells.

Polyfunctionality plot showing the composition of NK subsets secreting 0, 1, 2, 3, 4, 5, and > 5 cytokines in licensed (red) and unlicensed (blue) NK cell subsets. The frequency for each major category is shown as indicated.

Figure 2-19

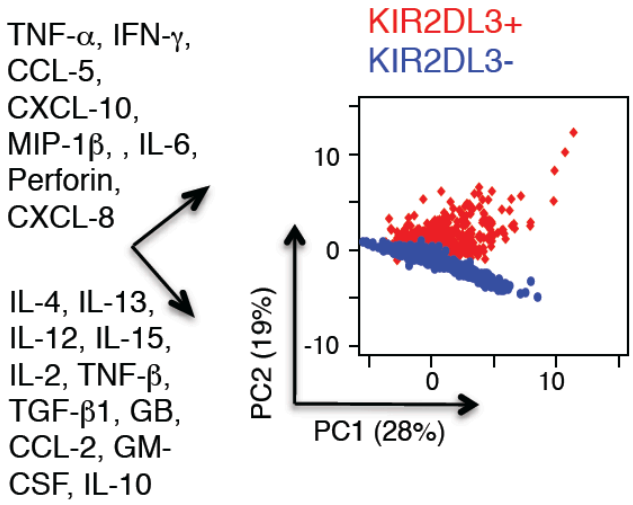

Figure 2-19. Licensed NK cells from an AA haplotype individual display higher effector protein production capacity.

PCA of secretion profiles from licensed (red) and unlicensed (blue) NK subsets. Percentage of variation explained by each component is shown in parentheses. The factors for each component are indicated left of the plot.

Figure 2-20

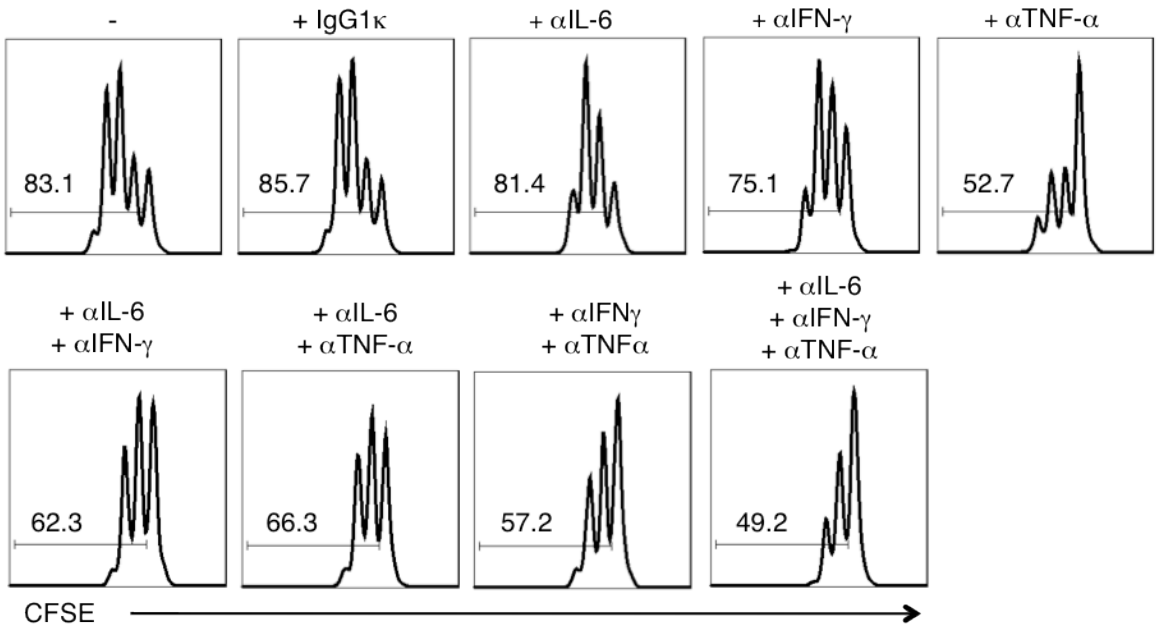

Figure 2-20. Neutralizing IFN-γ, TNF-α, and IL-6 in NK-T cell co-culture ameliorated $CD4^+$ T cell proliferation.

NK and autologous $CD4^+$ T cells were isolated from an AA haplotype licensed individual, stimulated with 1.5 μg mL^{-1} immobilized anti-CD3 and anti-CD28, and co-cultured in 2ng mL^{-1} (26 I.U) IL-2 for 72 hours. Histograms of $CD4^+$ T cell CFSE dilution without or with the indicated neutralizing antibodies. The number in each histogram indicates the percentage of cells proliferated.

Figure 2-21

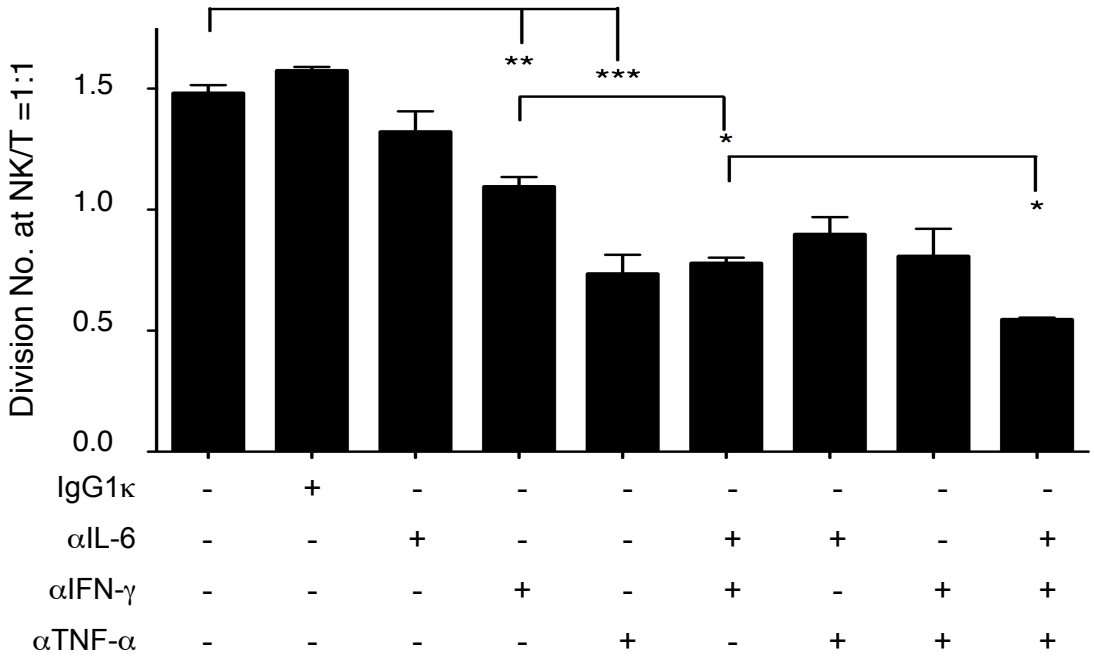

Figure 2-21. Neutralizing IFN-γ, TNF-α , and IL-6 in NK-T cell co-culture ameliorated $CD4^+$ T cell proliferation.

Bar plot of $CD4^+$ T cells division number at NK/T = 1:1 from the AA haplotype healthy individual. (Mean ± SEM, n = 2 to 6, two-tailed Student's t-test, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$).

Figure 2-22

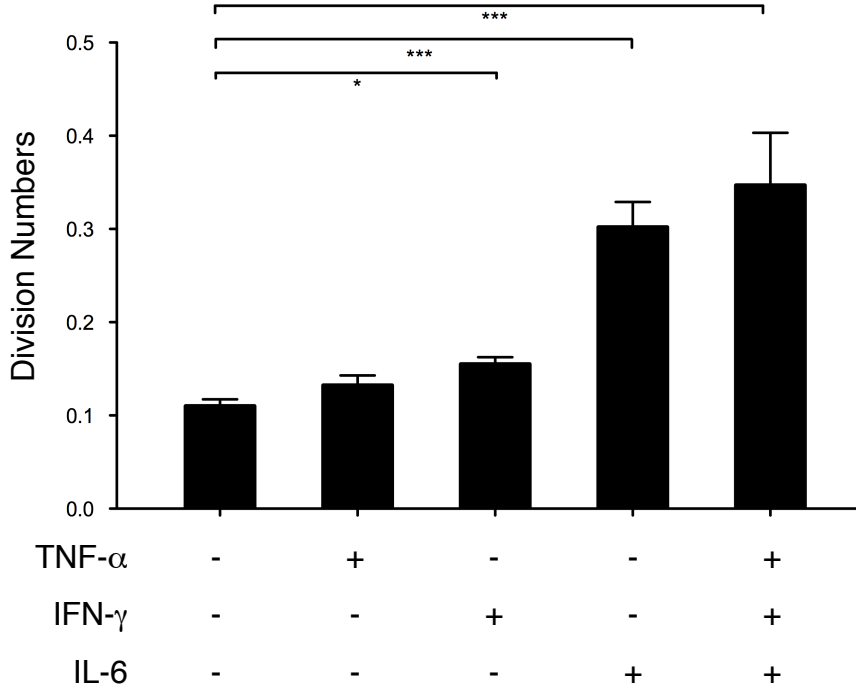

Figure 2-22. Addition of selected NK cytokines promoted CD4+ T cell proliferation.

Barplot of CD4+ T cell proliferation in 72-hour co-culture with or without of the indicated cytokines at the concentration of 20 ng ml^{-1}. Division numbers were calculated as Log_2 (mean of overall CFSE intensity / mean of undiluted CFSE intensity). n = 2 to 7, Student's t-test, two-tailed, (* $p<0.05$, ***$p < 0.0001$).

Figure 2-23

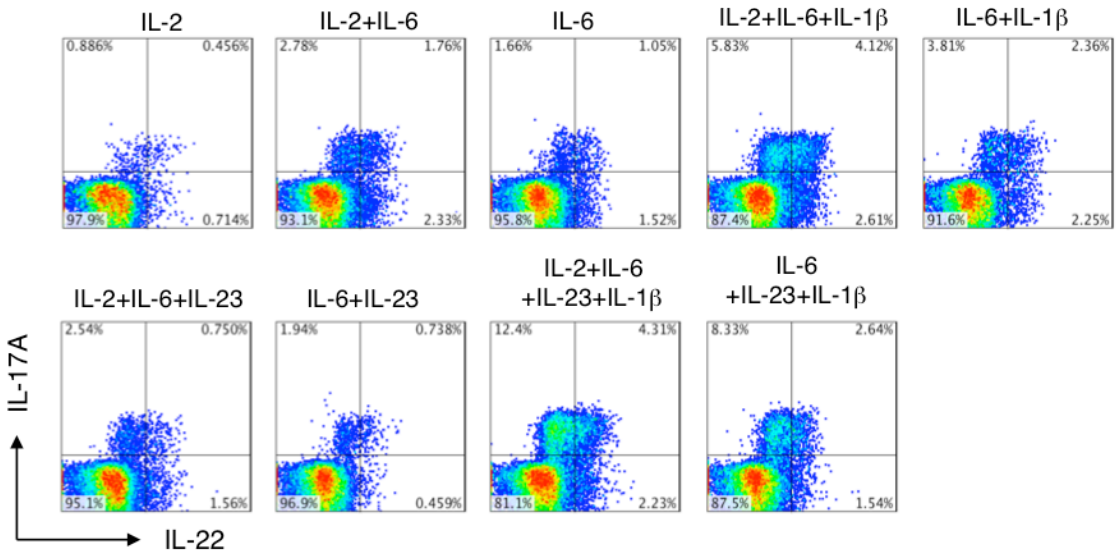

Figure 2-23. *in vitro* Th17 differentiation in various cytokine combinations.

Scatter plot of IL-17A and IFN-γ intracellular production from CD4+ T cells at D14 after differentiation with or without IL-2 at 2ng mL^{-1}, in the presence or absence of IL-1β, IL-23, or IL-6, at 50ng mL^{-1}, as indicated.

Figure 2-24

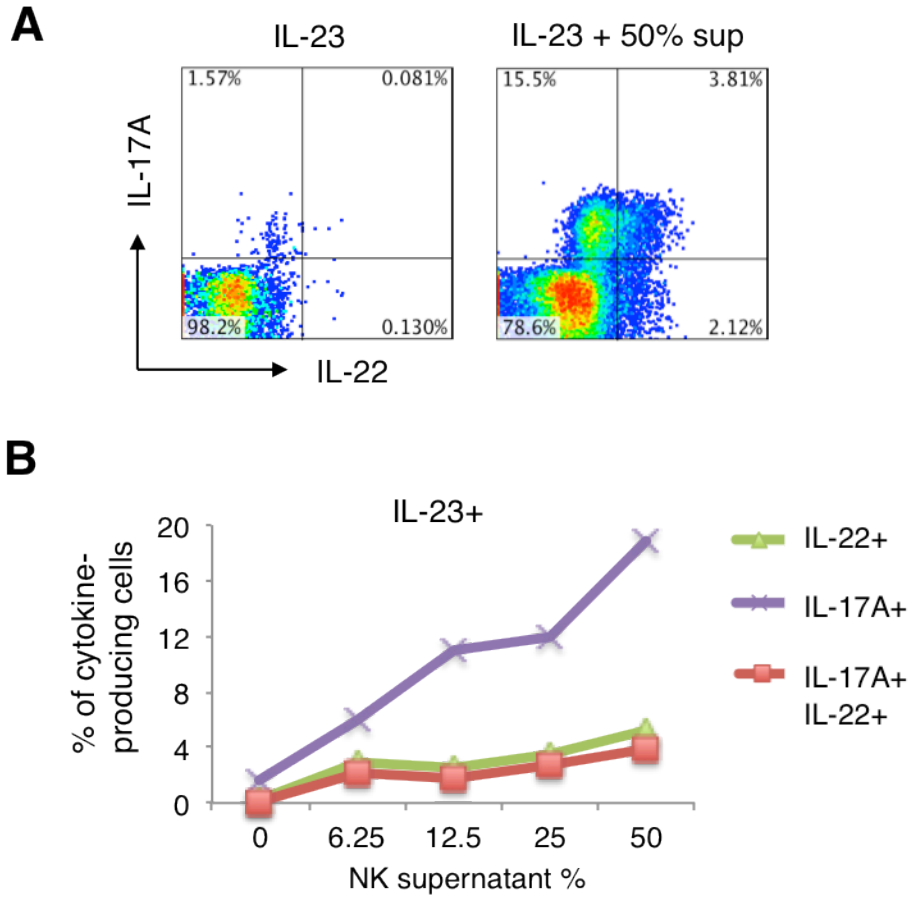

Figure 2-24. Supernatant of licensed NK cells drives human T_H17 cells differentiation synergistically with IL-23 *in vitro*.

Freshly isolated $CD4^+$ T cells from healthy subjects were stimulated with 1μg mL^{-1} immobilized anti-CD3 and 0.2μg mL^{-1} soluble anti-CD28, cultured in the presence of indicated cytokine with or without licensed NK cell 72-hour culture supernatant for 6-7 days. $CD4^+$ T cells were expanded for another 6-7 days in the same condition, and with 2ng mL^{-1} (26 I.U) IL-2. A Left panel: 2D scatter plot of IL-17A and IL-22 intracellular production with IL-1β or IL-23 at 50ng mL^{-1}, in the presence or absence of licensed NK cell supernatant. Numbers in each quadrant represents the percentage of cell in that

quadrant. (B) Line plot of the frequencies of IL-22^{+} (green triangle), IL-17A^{+} (purple cross), and IL-17A^{+}IL-22^{+} (red square) populations at different amounts of licensed NK supernatant. The licensed NK cells were isolated from licensed donor #0711, who is AA haplotype but also contains KIR2DL2. This result is representative of three independent experiments.

Figure 2-25

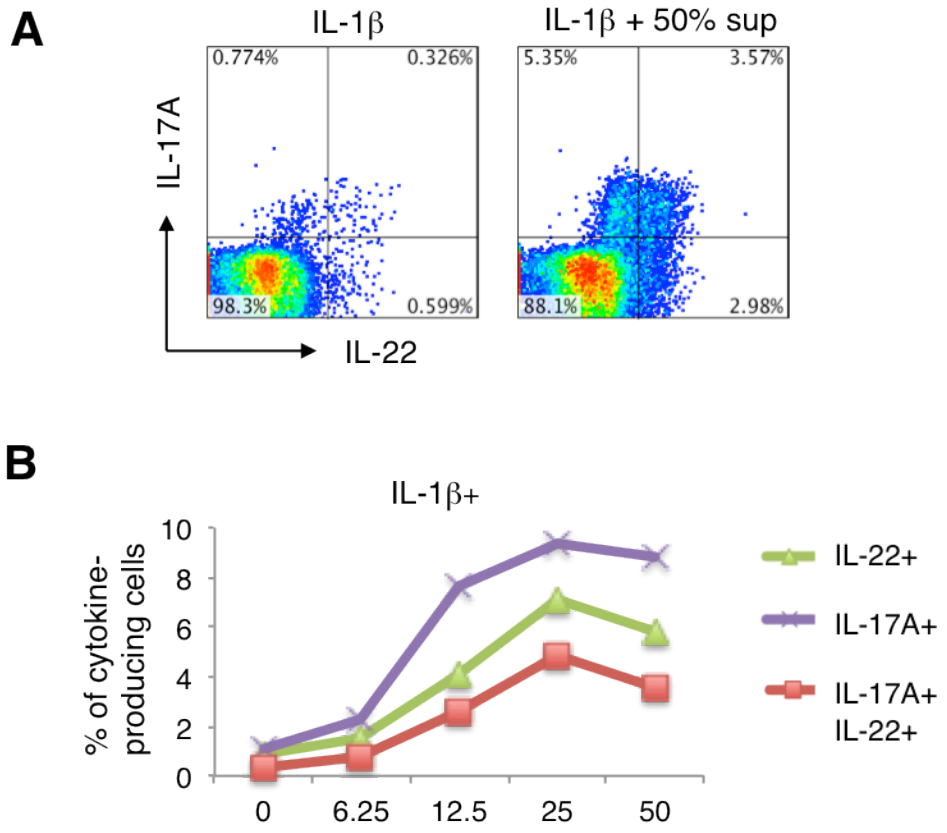

Figure 2-25. Supernatant of licensed NK cells drives human T_H17 cells differentiation synergistically with IL-1β *in vitro.*

A Left panel: 2D scatter plot of IL-17A and IL-22 intracellular production with IL-1β or IL-23 at 50ng mL^{-1}, in the presence or absence of licensed NK cell supernatant. Numbers in each quadrant represents the percentage of cell in that quadrant. (B) Line plot of the frequencies of IL-22^+ (green triangle), IL-$17A^+$ (purple cross), and IL-$17A^+$IL-22^+ (red square) populations at different amounts of licensed NK supernatant. The licensed NK cells were isolated from licensed donor #0711, who is AA haplotype but also contains KIR2DL2. This result is representative of three independent experiments.

Figure 2-26

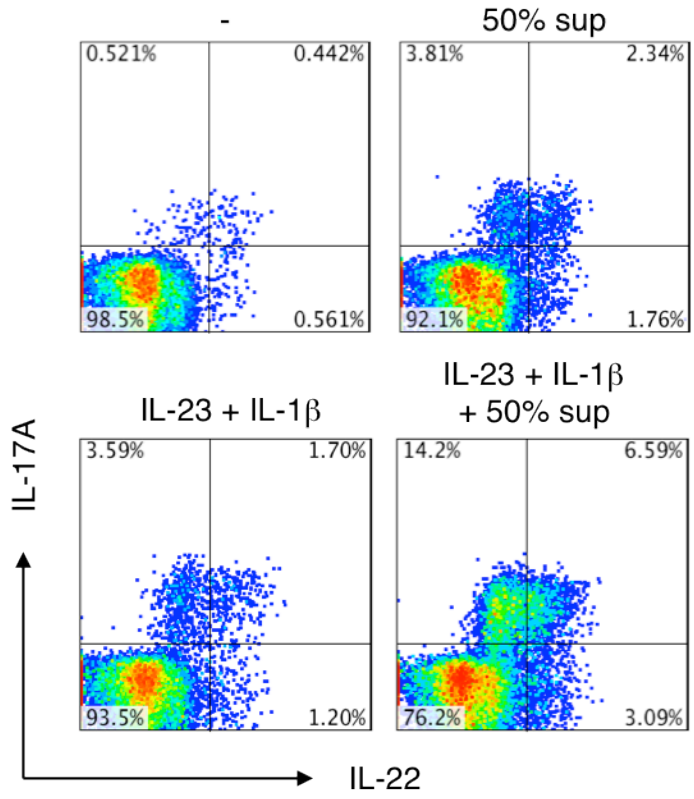

Figure 2-26. Licensed NK cell supernatant facilitated in vitro Th17 differentiation alone or synergistically with IL-1β and IL-23.

Scatter plot of IL-17A and IFN-γ intracellular production from CD4+ T cells at D14 after differentiation with IL-1β or IL-23 and IL-1β, at concentration of 50ng mL^{-1}, with or without the addition of NK cell supernatant. Numbers in each quadrant represents the percentage of cell in that section.

Figure 2-27

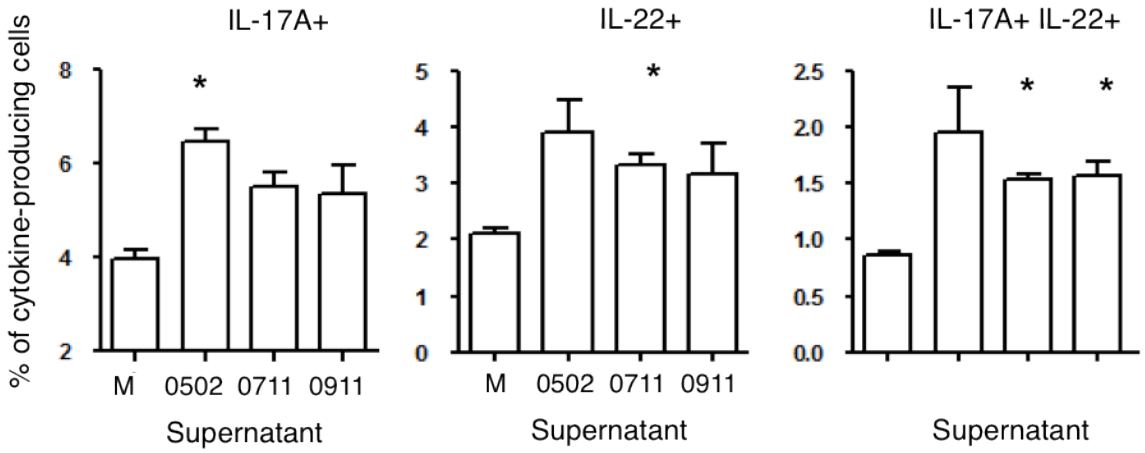

Figure 2-27. Supernatant of NK cells from licensed healthy individuals consistently facilitated Th17 differentiation *in vitro*.

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 control media or 50% NK cell supernatants from four different licensed healthy donors. M stands for negative control media, is NK culture media without NK cells. Assays were conducted in the presence of 50ng mL^{-1} IL-23. ((Mean ± SEM, n = 2, two-tailed Student's t-test, * $p < 0.05$).

Figure 2-28

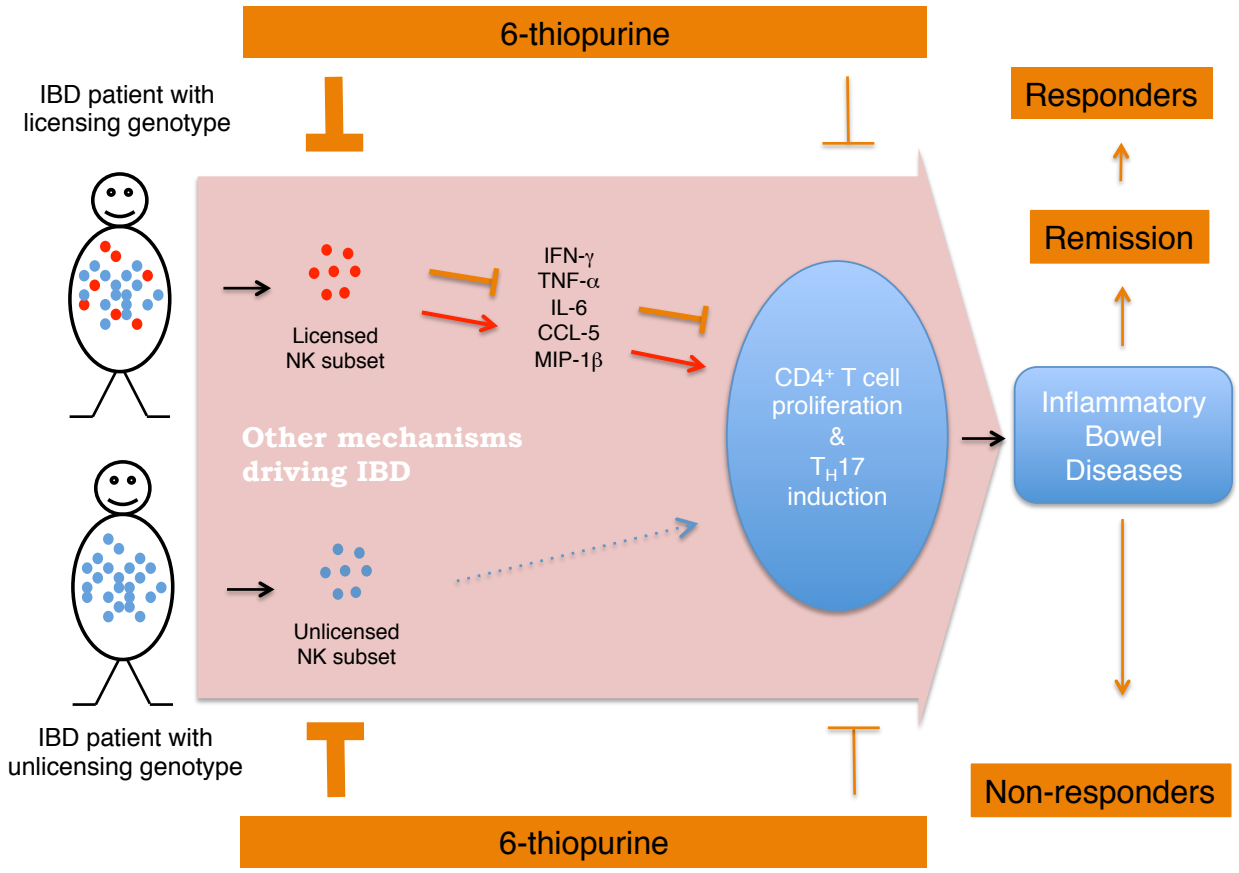

Figure 2-28. The mechanism of using KIR-HLA genetics as a predictor for thiopurine responsiveness.

Individuals with licensing genotypes (AA haplotype with HLA-C1C1 or HLA-Bw4/4) have licensed NK cell subset, which are stronger producers for IFN-γ, TNF-α , IL-6, CCL-5, MIP-1β, et al. than unlicensed NK cell subset. These pro-inflammatory mediators promote CD4+ T cell proliferation and TH17 cell induction. In conjunction with other mechanisms (shown as the pink background) that are known to drive IBD pathogenesis, licensed NK cells add to another level of susceptibility. Thiopurine diminishes lymphocyte functions by inhibiting Rac1 signaling, and preferentially eliminates NK cells (indicated by the thick orange arrow towards NK cells, and the thin arrow towards CD4

T cells). Thus, in either type of IBD patients, NK cells are targeted, but only in patients with licensed NK cells, thiopurine effectively dampen the pro-inflammatory cues from NK cells, and subsequently more effectively induce remission. Therefore, patients with licensing genotypes are more responsive to thiopurine than patients with unlicensing genotypes.

Table 2-1. Healthy Donor and Crohn's Disease Patient Demographics

		Healthy	Crohn's Patients
Total healthy donors		11	20
Gender	Female	7	12
	Male	4	8
Age (average ± SD)		27.3 ± 3.9	49.5 ± 9.3
KIR genotype	Non AA	8	0
	AA	3	20

AA genotypes: Carriers of two A haplotypes, which contains a fixed number of nine KIR genes (3DL3-2DL3-2DP1-2DL1-3DP1-2DL4-3DL1-2DS4-3DL2); non AA genotype: Carriers of A and B haplotypes or carriers of two B haplotypes, which contains one or more B haplotype-specific genes (2DL2, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5 and 3DS1).

Table 2-2. Hierarchy in the strength of NK cell education (adapted from [83])

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	-

Table 2-3. HLA allotype distribution and Chi2 test of AA haplotype CD Patients

% (No.)	C1C1	C1C2	C2C2	Chi^2 Test
Bw6/6	15.8 (72)	11.9 (54)	3.7 (17)	
Bw4/6	12.7 (58)	27.7 (126)	11.4 (52)	*** P < 0.0005
Bw4/4	4.0 (18)	8.4 (38)	4.4 (20)	

Values are % of patients or (absolute number of patients)

Table 2-4. Media protein biomarker and single cell cytokine panel

Antibody	Manufacturer	Initial panel	Finalized panel	Single cell panel
IFN-γ	R&D	+	+	+
TNF-α	eBioscience	+	+	+
Granzyme B	R&D	+	+	+
Perforin	Abcam	+	+	+
TNF-β	R&D	+	+	+
TGF-β1	R&D	+	+	+
IL-2	R&D	+	+	+
IL-4	eBioscience	+	+	+
IL-6	R&D	+	+	+
IL-10	R&D	+	+	+
IL-12	R&D	+	+	+
IL-13	R&D	+	+	+
IL-15	R&D	+	+	+
CCL-5	R&D	+	+	+
GM-CSF	eBioscience	+	+	+
MIP-1β	R&D	+	+	+
CCL-2	R&D	+	+	+
CXCL-8	R&D	+	+	+
CXCL-10	R&D	+	+	+

IL-1β	eBioscience	+	-	-
IL-5	R&D	+	-	-
IL-7	R&D	+	-	-
IL-17A	eBioscience	+	-	-
IL-17E	R&D	+	-	-
IL-17F	R&D	+	-	-
IL-22	R&D	+	-	-
TGF-β2	R&D	+	-	-
CCL-11	R&D	+	-	-
CXC-L3	R&D	+	-	-
CCL-21	R&D	+	-	-
MIP-1α	R&D	+	-	-

*The single cell barcode chip can address 19 cytokine analytes. Among the 31 cytokine initially assessed for NK production, 24 were detected above threshold, although the detectable number of cytokines was lower in any single individual. To select the cytokines for the final panel: (a) we excluded MIP-1α (its production was highly concordant with MIP-1β; (b) we included IL-2 and IL-13, which were readily detectable in many individuals and of substantial IBD immunologic interest; and, (c) we excluded several because they were infrequently above detection (IL-1β, IL-5, IL-7, IL-17 (A, E, F), IL-22, CCL-11, CCL-21, CXCL-1, TGF-β2).

Table 2-5. HLA allotype distribution in total and AA haplotype MS Patient

A. Total MS Patients

No.	C1C1	C1C2	C2C2	Total
Bw6/6	12	9	2	23
Bw4/6	1	18	2	21
Bw4/4	0	2	4	6
Total	13	29	8	50

B. AA haplotype MS Patients

No.	C1C1	C1C2	C2C2	Total
Bw6/6	2	4	0	6
Bw4/6	0	10	0	10
Bw4/4	0	0	1	1
Total	2	14	1	17

Values are absolute number of patients.

Table 2-6. Thiopurine IBD Cohort Demographics

		CD	UC	Total IBD
Total healthy donors		106	28	134
Gender	Female	52	19	71
	Male	54	9	63
Age (average ± SD)		11.2 ± 3.5	10.8 ± 4.9	11.1 ± 3.8
KIR genotype	Non AA	76	19	95
	AA	30	9	39
Steroid-free Remission at week 26	In Remission	54	9	63
	Not in Remission	52	19	71

Table 2-7. The distribution of thiopurine-induced steroid-free remission at week 26 in licensed vs. unlicensed subsets of IBD pediatric cohort

AA haplotype patients			
	Remission	Not in Remission	P value
Licensed	62.5% (10)	37.5% (6)	0.016
Unlicensed	20.0% (4)	80.0% (16)	
All patients			
	Remission	Not in Remission	P value
Licensed	56.1% (32)	43.9% (25)	0.287
Unlicensed	44.1% (30)	55.9% (38)	

Values are % of patients in remission (patients in remission)

P values were generated by Fisher's exact test for small size samples.

Licensed: HLA-C1C1 or HLA-Bw4/Bw4 IBD pediatric patients

Unlicensed: Other IBD pediatric patients

CHAPTER 3

Genetic Licensing Uncovers a Role for NK Cells in Immune Colitis by Co-activation of $CD4^+$ T Cells

Abstract

Background & Aim

Natural Killer cells are members of the innate immune system mainly known for their cytolytic abilities. They are divided into distinct subsets comprised of licensed and unlicensed NK cells. NK cells are programmed at a genetic level to express discriminating Killer Immunoglobulin Receptors (KIRs) in humans and Ly49 receptors in mice. The engagement of these receptors by its cognate HLA ligand induces differentiation of NK cells into a licensed subset, which has functional properties distinct from the unlicensed types. In humans, evidence suggests that genetic licensing is a risk factor for Crohn's disease and recent work (L Lin et al) uncovered diverse cytokine production states programmed by genetic licensing. However, limited and discrepant studies exist on the relationship of NK cells to immune mediated colitis. To biologically test this genetic association, this study investigates the interaction between murine licensed NK cells and $CD4^+$ T cells which are well known mediators of inflammation in mouse colitis models.

Methods

We used co-culture assays and flow cytometry to study the impact of bulk or sorted spleen NK cells from C57/BL6 mice on $CD4^+$ T cell proliferation. We investigated the nature of the interaction between licensed NK cells with $CD4^+$ T cells utilizing transwell assays. Utilizing microfluidic and Luminex platforms, we analyzed the cytokine profiles of NK cell secretion in 72-hour culture. We also neutralized co-stimulatory molecules expressed by licensed NK cells in co-cultures, or added back cytokines

secreted by licensed NK cells to $CD4^+$ T cell culture to validate the functions of candidate mediators.

Results

Here we show that NK cells from C57/BL6 mice augment $CD4^+$ T cell proliferation *ex vivo*, with the licensed subset being more capable than unlicensed subset. Multiplex cytokine analysis of licensed NK cell media also demonstrate higher levels of IL-6, IFN-γ, and TNF-α production compared to unlicensed NK cells. This cytokine profile mirrors the cytokine secretion profile of licensed human NK subsets. In contrast to licensed human NK cells however, transwell studies showed that licensed NK cell stimulation of $CD4^+$ T cells also occurred in a contact dependent manner. Antibody blockade of candidate receptor-ligand interactions (OX40-OX40L, CD28-CD86, CD28-CD80) between NK and $CD4^+$ T cells diminished the stimulatory effect of NK cells. Adding back cytokine produced by licensed NK cells enhanced the proliferation of $CD4^+$ T cells.

Conclusion

Our results demonstrate that licensed murine NK cells augment $CD4^+$ T cell proliferation and this may be mediated by increased secretion of soluble mediators such as IL-6 and TNF-α, as well as cell-to-cell contact dependent mechanism. This feature of murine licensed NK cells is different from the solely cytokine-mediated stimulation mechanism seen in human licensed NK cells.

Introduction

NK cells licensing depends on KIR and HLA genotype

NK cells comprise 5 to 20% of peripheral blood mononuclear cells in humans and play an important role primarily in defense against viral infections and tumor surveillance. A sophisticated repertoire of cell surface receptors control their activation, proliferation, and effector functions.[30] Of these receptors, much attention has been focused on killer immunoglobulin-like receptors (KIRs) which can inhibit or activate NK cells, depending on the structure of the molecules.[23,30] There are 14 KIR genes in total - six genes encode for activating receptors (2DS1-2DS5 and 3DS1) while seven encode for inhibitory receptors (3DL1-3DL3, 2DL1- 2DL3, 2DL5). One gene encodes KIR2DL4, which has both inhibitory and activating functions.[3,84] An individual may carry any number and combination of KIR genes, and simultaneously express several different KIRs stochastically on NK cells.[51]

Major histocompatibility class (MHC) I molecules are the only known ligands for KIRs, and each inhibitory KIR cognizes a specific HLA class I ligand: for example, inhibitory KIR2DL1 recognizes HLA-C2 allotypes while inhibitory KIR2DL2 and KIR2DL3 recognize HLA-C1 allotypes.[3] Other inhibitory KIRs, such as 3DL1, interact with HLA-B allotypes that have a Bw4 motif. The interaction between inhibitory KIRs and their cognate HLA class I ligands are especially significant because it induces NK cell differentiation into a phenotype with enhanced cytotoxicity and cytokine producing ability. This differentiation is termed “licensing” and the resultant functionally enhanced NK cells are termed “licensed” NK cells.[23,39,51] Licensing is a characteristic of NK cells pre-determined by the combination of KIR and HLA genetics. KIR gene composition

may vary extensively between individuals. Individuals who lack either inhibitory KIR genes or cognate HLA ligands are considered KIR-HLA mismatch.[51,85] Individuals with KIR-HLA mismatch or weakly binding KIR-HLA interaction have weakly licensed or unlicensed NK cells.

Licensed NK cells are high output producers of inflammatory cytokines and contribute to pathogenesis of IBD

Given KIR specificity for HLA allotypes, it is not surprising that KIR gene variations affect susceptibility to a number of diseases. A growing body of evidence illustrates the influence of KIRs in autoimmune diseases such as psoriatic arthritis, scleroderma, and type I diabetes.[70,86] The role of KIR genes in inflammatory bowel disease has also been shown: several studies show that KIR2DL2/ KIR2DL3 heterozygous individuals who express its cognate ligand HLA-C1 have increased susceptibility to Crohn's disease.[10,38] Studies from our lab validate the association of KIR2DL3 genes and HLA-C1 ligand expression with increased susceptibility to Crohn's disease (Lin L et al).

Following the confirmation of association between KIR genes and IBD susceptibility, our lab investigated the immunologic mechanism underlying KIR-mediated NK cell licensing and IBD pathogenesis. Our studies show that licensed KIR2DL3^{+} NK cells isolated from IBD patients are more potent stimulators of CD4^{+} T cell proliferation compared to unlicensed KIR2DL3^{-} NK cells. Licensed NK cells also drive CD4^{+} T cell polarization towards T_H17 cells, a signature colitogenic CD4^{+} T helper cell subset. Multiplexed cytokine study of CD cohorts demonstrates that genetically

licensed and unlicensed NK cells exhibit consistent and distinct cytokine profiles, with licensed NK cells distinguished by high-output, pro-inflammatory, and poly-cytokine expression. Select cytokines among this output account for the unique capacity of licensed NK cells to efficiently augment antigenic $CD4^+$ T cell proliferation and T_H17 polarization. These results demonstrate the novel significance and immunologic mechanism of licensed NK cells in IBD pathogenesis.

In this study, to biologically test the contribution of the licensing genetics in murine immune colitis, we investigated the interaction between murine licensed NK cells and $CD4^+$ T cells. We showed that licensed NK cells from C57/BL6 mice augmented $CD4^+$ T cell proliferation *ex vivo*. Multiplex cytokine analysis of licensed NK cell media also demonstrated higher levels of IL-6, IFN-γ, and TNF-α production compared to unlicensed NK cells. This cytokine profile mirrors the cytokine secretion profile of licensed human NK subsets. In contrast to licensed human NK cells, transwell studies showed that murine licensed NK cell stimulation of $CD4^+$ T cells also occurred in a contact dependent manner. Antibody blockade of candidate receptor-ligand interactions (OX40-OX40L, CD28-CD86, CD28-CD80) between NK and $CD4^+$ T cells diminished the stimulatory effect of NK cells. Adding back IL-6, IFN-γ, and TNF-α enhanced the proliferation of $CD4^+$ T cells, confirming the stimulatory effects of licensed NK cell secretion.

Materials and Methods

Mice

C57BL/6 and β2m$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME), and were housed under specific pathogen-free conditions at the animal facilities of the UCLA Division of Laboratory Animal Medicine (Los Angeles, CA), in accordance with the Institutional Animal Care Committee (ARC) guidelines. Age and gender-matched mice were used for experiments.

Splenocyte Preparation

Single-cell suspensions from spleen were prepared as described.[87,88] Briefly, spleens were smashed and filtered through 70 micro strainers to extract splenocytes. RBCs were then lysed (ACK Lysis buffer, Lonza, Rockland, ME) and WBCs were washed once with medium (RPMI 1640; Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) FBS (Mediatech, Manassas, VA), 1% (v/v) penicillin-streptomycin-glutamine (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, 29.2 mg/ml L-glutamine; Invitrogen), and 50 μM β-mercaptoethanol (Sigma-Aldrich).

Cell isolation

Spleen NK cells were isolated using Mouse NK cell negative selection kit (StemCell Technologies, Vancouver, BC, Canada). Mouse CD4 T cells were purified from spleen using Mouse T cell enrichment kit and CD4 positive selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated NK cells and T cells were checked to be above 90%.

NK-T cell co-culture and blocking assays

Before co-culture, round bottom 96-well plates were coated with anti-CD3/CD28 antibody (R&D Systems, Minneapolis, MN) in PBS at 1.5μg ml^{-1} at room temperature for 2 hours, or at 0.5μg ml^{-1} at 4ºC overnight. $CD4^+$ T cells were stained with 0.2μM CFSE (Invitrogen, Carlsbad, CA), and co-cultured with NK cells for 72 hours in 96-well plates at 1 x 10^6 cells ml^{-1} with complete RPMI 1640 medium (recipe provided above). Blocking antibodies for mouse OX40L, CD86, and CD80 (eBioscience, San Diego, CA) were added to the co-culture at concentration of 5μg ml^{-1}. Neutralizing antibodies, for IL-6, IFN-γ, TNF-α, and isotype control IgG1κ (eBioscience, San Diego, CA), and their combinations were added to the co-culture at 4μg ml^{-1}. The recombinant cytokines IL-6, IFN-γ, and TNF-α (eBioscience, San Diego, CA) was each added to a final concentration of 20 ng ml^{-1}, comparable to the concentration measured for these cytokines in NK cell 72-hour 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 $CD4^+$ T cells were placed on the plate side of the transwell.

Multiplex cytokine ELISA assays

Mouse NK cells were cultured for 72 hours in round-bottom 96-well plate at 1 x 10^6 cells ml^{-1} in 2ng ml^{-1} IL-2 with complete RPMI 1640 medium, containing 10% Fetal Bovine Serum (FBS), 100 I.U ml^{-1} penicillin 100 μg ml^{-1} streptomycin, 10mM HEPES buffer, 2 mM glutamine (Cellgro, Manassas, VA), and 5 x 10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). Then media samples were collected, stored at -80^oC. Before

analysis, samples were thawed, concentrated, and assayed as one batch. The NK secretion antibody panel was chosen to incorporate non-redundant secretions detectable at NK 72-hour culture based on our previous discover made on human NK cells.

Flow Cytometry and Cell sorting

Antibodies against mouse CD3, CD4, CD19, Ly49A, Ly49C/I, and NKG2D were purchased from eBioscience (San Diego, CA). Cell staining for flow cytometry were performed as reported previously.[88] Phenotypic analysis of NK cell phenotype and proliferation was performed using flow cytometry after staining of cells with fluorescence dye–conjugated antibodies. Labeled cells were analyzed with a FACSCalibur flow cytometer using CellQuest software, or LSR II (BD Biosciences, San Jose, CA) using FACSDiva software (BD Biosciences, San Jose, CA) at UCLA Flow Cytometry Core, and data analysis was performed using FlowJo (Tree Star Inc., Ashland, OR). Cells were sorted for $CD3^-CD19^-$ $Ly49C/I^+$ $Ly49A^-$ and $CD3^-CD19^-$ $Ly49C/I^-$ $Ly49A^+$, and $CD3^-$ $CD19^-$ $Ly49C/I^-$ $Ly49A^-$ using Aria I equipped with FACSDiva software (BD Biosciences, San Jose, CA).

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Comparisons were drawn using a 2-tailed Student's t-test or an analysis of variance test. P values < .05 were considered significant and are indicated as follows: *P < .05, **P < .01, and ***P < .001. Each experiment was repeated.

Results

Murine Ly49 family

The functional homologue of human KIR family is the murine Ly49 family (Figure 3-1). Since C57/BL6 mice express H-2D^{d} and H-2K^{b} (binding to Ly49A and Ly49C/I with intermediate and strong licensing effects, respectively), their NK cells are licensed and will be used as donors for licensed NK cells. On the other hand, b2m$^{-/-}$ KO mice don't express MHC class I molecules, and they will be used as donors for unlicensed NK cells.

C57BL/6 mouse NK cells potently enhance CD4 T^{+} cell proliferation

It has been shown in the human that licensed NK cells facilitate CD4^{+} T cell proliferation, here we examined whether murine licensed NK cells have a similar impact on CD4^{+} T cells. We co-cultured spleen NK cells with CD4^{+} T cells in a 72-hour co-culture with weak anti-CD3 anti-CD28 activation, and examined the proliferation of CD4^{+} T cells afterwards. We observed that CD4^{+} T cells proliferated in the absence of NKs, and increased proliferation when there were more NK cells in the co-culture (Figure 3-2A We calculated the CD4^{+} T cell division index (DI) at each NK: T ratio, generated by the proliferation function of flowJo. CD4^{+} T cell DI follows a good linear correlation (R^2= 0.999) with NK: T ratio, indicating NK facilitation of CD4^{+} T cell proliferation is dose-dependent (Figure 3-2B).

NK cells from C57BL/6 mice are more potent producers of IL-6, TNF-α, and IFN-γ

Based on the contact-independent mechanism of NK action observed in human, we collected murine C57BL/6 and $\beta 2m^{-/-}$ NK cell 72-hour culture, and profiled for ten mouse protein mediators, using integrated blood barcode chip (IBBC). Licensed NK cells (from C57BL/6 mice) produced significantly higher levels of pro-inflammatory cytokines, including IFN-γ ($p=0.007$), IL-6 ($p=0.02$), and TNF-α ($p=0.004$) (Figure 3-3), then unlicensed NK cells from $\beta 2m^{-/-}$ mice. It's worth mentioning that because we did not use a pan NK cell marker for sorting, the $Ly49C/I^{-}Ly49A^{-}$ subset also contained non-NK cells, and its cytokine secretions are not strictly representative of the true $Ly49C/I^{-}$ $Ly49A^{-}$ NK cells. For the following sortings, we had included NKp46 as the pan NK cell marker, thus the $Ly49C/I^{-}Ly49A^{-}$ subset is strictly double negative NK cells.

The effect of licensed NK on $CD4^{+}$ T cell proliferation is partially contact-dependent

Although human licensed NK cells promote $CD4^{+}$ T cell proliferation almost in an exclusively contact-independent manner, we wanted to see if murine licensed NK cells behave similarly. Therefore, segregated NK from $CD4^{+}$ T cells using a 0.1-micron pore size transwell, which only allows interchange of soluble mediators but not direct contact of the two populations. Surprisingly, $CD4^{+}$ T cell proliferation augmented by the presence of NK cells was slightly effected by transwell (Figure 3-4), indicating that besides soluble proteins secretion, murine licensed NK cell function was also mediated by direct interaction with $CD4^{+}$ T cells.

The effect of licensed NK on $CD4^+$ T cell proliferation depends co-stimulatory molecules.

NK cells express several T cell co-stimulatory molecules, including 2B4, OX40 ligand, et al. Therefore, we tested if the NK facilitation of $CD4^+$ T cell proliferation was mediated by these molecules. We treated the NK-T cell co-culture with blocking antibody against OX40 Ligand, CD80 (B7-1), or CD86 (B7-2), and there were notable reductions of $CD4^+$ T cell proliferation when OX40L or CD80 were blocked(Figure 3-5). This suggested that licensed NK cell effect depended on their expression of co-stimulatory molecules.

IFN-γ, TNF-α, and IL-6 account for the capacity of murine 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-$CD4^+$ T cell co-cultures (Figure 3-6). Neutralization of TNF-α alone or TNF-α and IFN-γ had modest effects, while neutralization of IFN-γ even had non-significant but slight promotion of $CD4^+$ T cell proliferation, possibly suggesting that TNF-α and IFN-γ are not essential for $CD4^+$ T cell proliferation in this particular *in vitro* setting. Neutralization of IL-6 alone or in combination with TNF-α, or both TNF-α and IFN-γ, markedly reduced $CD4^+$ T cell proliferation, indicating IL-6 is an effective driver for $CD4^+$ T cell proliferation. We further evaluated $CD4^+$ T cell proliferation in the absence of NK cells, in which exogenous cytokines were added at 20ng mL^{-1} (Figure 3-7). IFN-γ, TNF-α, IL-6 and their combinations all facilitated $CD4^+$ T cell proliferation at

various extents, but the addition of IL-6 and TNF-α had the greatest effect. These results indicated that IL-6 is both necessary and sufficient to augment $CD4^+$ T cell proliferation, while IFN-γ and TNF-α are sufficient but not necessary to augment $CD4^+$ T cell proliferation. 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.

Brief stimulation with low dose IL-15 increases NK cell viability and proliferation *in vitro*.

In order to investigate the effects of differentially licensed subsets of NK cells: Ly49C/I^+Ly49A, Ly49C/I^-Ly49A^+, and Ly49C/I^-Ly49A^-, we need to sort for these subsets. One of the challenges we have experienced is to maintain the viability of the NK subsets after going through extensive period of sorting. To increase the yield of functionally active NK cells, we incubated freshly isolated NK cells with suboptimal doses of IL-15 at 37°C for 2 hours,[89] and then put them on ice for 4 hours to mimic sorting conditions. After 96-hour culture, NK cells were stained with 7-AAD to test their viability and examined for their proliferation by CFSE titration assay. The percentage of dead cells dropped dramatically from 67.3% to 16.6% (***$p < 0.0001$) by incubating NK cells with 10 ng mL^{-1} IL-15 at 37°C for 2 hours, while further increasing the dose of IL-15 only had marginal effects (Figure 3-8). All the doses of IL-15 treatments resulted in NK cell proliferation, with 10 ng mL^{-1} IL-15 having the minimal proliferative effect (Figure 3-9). These results indicate that brief incubation with 10 ng mL^{-1} IL-15 before sorting helps to maintain the viability of NK cell while proliferate NK cells at the leastextent.

Sorted Ly49C/I single positive NK cells are the most potent to facilitate $CD4^+$ T cell proliferation compared to other NK cell subsets.

Based on the previous results, here we examined the impact of the different sorted NK cell subsets on $CD4^+$ T cell proliferation after brief stimulation with 10 ng mL^{-1} IL-15. $CD4^+$ T cell alone had a division index of 0.29, and the presence of any NK cell subset had a positive impact on $CD4^+$ T cell proliferation. The sorted Ly49C/I^+Ly49A^- subset (strongly licensed) had the highest capability in promoting $CD4^+$ T cell proliferation, while Ly49C/I^-Ly49A^- (*$p<0.05$) and Ly49C/I^-Ly49A^+ have lower capability. Due to the limited availability of NK cells after sorting, we were only able to obtain enough Ly49C/I^-Ly49A^+ NK cells for a single well of co-culture, but we will use more mice to harvest enough cells for triplicates for the following repeating experiments.

Discussion

In human, the genetic presence of strong NK licensing KIR/ligand pairs (KIR2DL3/HLA-C1 or KIR3DL1/HLA-Bw4) affects several important chronic inflammatory diseases: elevated susceptibility to CD, Celiac disease, spondyloarthropathy, psoriatic arthritis; enhanced resolution of Hepatitis C virus (HCV) infection, and slower progression in HIV-1 infection.[70-76] Understanding the nature of NK licensing contribution to disease susceptibility or protection is crucial for developing diagnostic and treatment strategies (Figure 2-28). Lin L et al. uncovered KIR-mediated licensing as a major mechanism to reprogram NK cell cytokine capacity; in accord with the distinct cytokines produced by licensed NK cells, those NK cells have the capacity to augment $CD4^+$ T cell activation and T_H17 differentiation, which provided a mechanistic basis for their genetic association to chronic inflammatory diseases.

Our findings from murine models largely validated the discoveries made in healthy individuals and Crohn's Disease patients. NK cells from C57/BL6 mice augment $CD4^+$ T cell proliferation *in vitro* through their enhanced capability of producing pro-inflammatory cytokines, including IFN-γ, TNF-α, and IL-6. On the other hand, distinct from the human counterpart, murine licensed NK cells also depend on direct contact to facilitate $CD4^+$ T cell proliferation (Figure 3-4). Blockade of candidate co-stimulatory molecules expressed by NK cells modifies the impact of licensed NK cells (Figure 3-5). This functional difference between mice and human NK cells and the underlying molecular mechanisms remain to be elucidated.

To validate if the differentially secreted cytokines by licensed NK cells, including IFN-γ, TNF-α, and IL-6 indeed drive $CD4^+$ T cell proliferation, we either neutralized

these cytokines from NK-$CD4^+$ T cell co-cultures, or added back these cytokines to $CD4^+$ T cell cultures. Cytokine addition results showed that IFN-γ, TNF-α, or IL-6 alone was sufficient to promote $CD4^+$ T cell proliferation. Cytokine neutralizing results indicates that IFN-γ and TNF-α might not be necessary to drive $CD4^+$ T cell proliferation, while blocking IL-6 alone or together with TNF-α, or both IFN-γ and TNF-α eliminates the impact of licensed NK cells. These observations do not exactly align with what was observed in the human system or the cytokine addition results; we suspect this could either be derived from the intrinsic differences between murine and human immune systems, or the artificial effects from introducing IgG into the co-culture. To eliminate the antibody-induced artifact, we plan to precipitate IgG by protein G beads after neutralizing the cytokines and before co-culture.

One major aim of this study is to model the role of NK cell licensing in immune-mediated colitis. We are currently challenged by the low abundance of licensed NK cells in mice and their reduced viability after sorting. We have been trying to increase the yield of functionally active NK cells by stimulating them with suboptimal dose of IL-15 briefly before sorting, which increased the viability of NK cells for more than four times. However, in order to harvest sufficient number of functionally active licensed NK cells for *in vivo* studies, we still need to validate the activity of IL-15 treated licensed NK cells through interaction with $CD4^+$ T cells and their cytokine secretion.

To investigate the role of licensed NK cells immune colitis, we will introduce sorted licensed NK cell subsets into $il2rg^{-/-}rag2^{-/-}$ recipient mice with $CD4^+CD45RB^{hi}$ T cell-induced colitis. Recipient mice will be monitored over time for their body weight

change, sacrificed when colitis manifests, and scored for histology and immunological trait characterization.

Figures and Tables

Figure 3-1

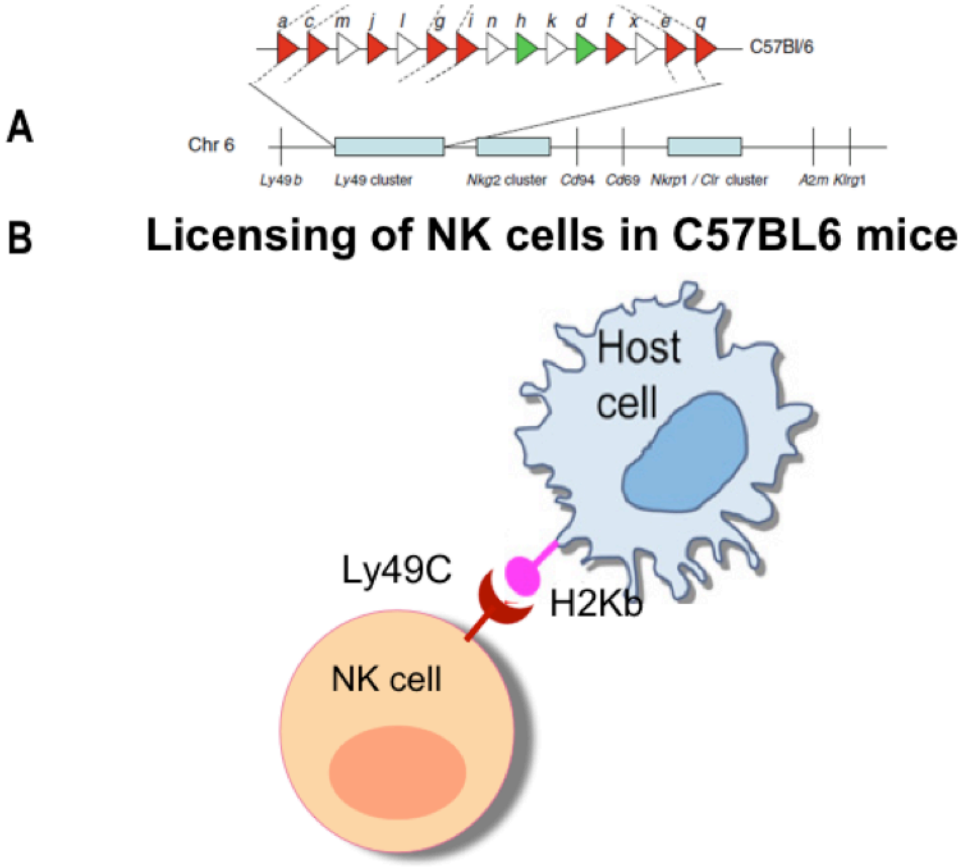

Figure 3-1. Ly49 cluster in mouse genome and NK cell licensing

(A) The Ly49 genes of C57BL6 mice. Red represents inhibitory receptors enabling licensing in presence of cognate HLA ligand. (B) Binding of Ly49C receptor to ligand H^2K^b results in the licensing of NK cell.

Figure 3-2

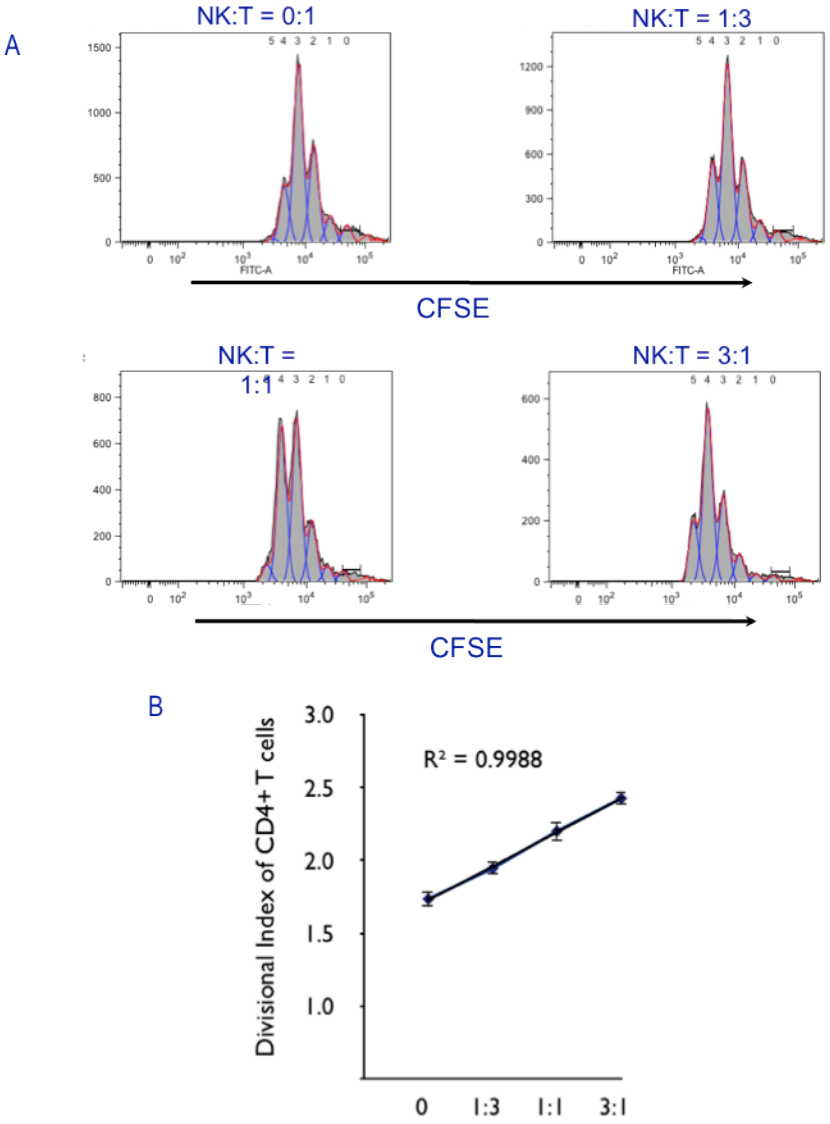

Figure 3-2. NK cells from C57BL6 mice co-activate CD4$^+$ T cells leading to augmented proliferation.

(A) CD4$^+$ T cell proliferation measured by Carboxyflourescein succinimidyl ester (CFSE) dilutional assay, is increased in a NK dose dependent manner during 72 hour co-culture period. (B) Linear correlation between NK:T ratio and divisional index of CD4$^+$ T cells.

Figure 3-3

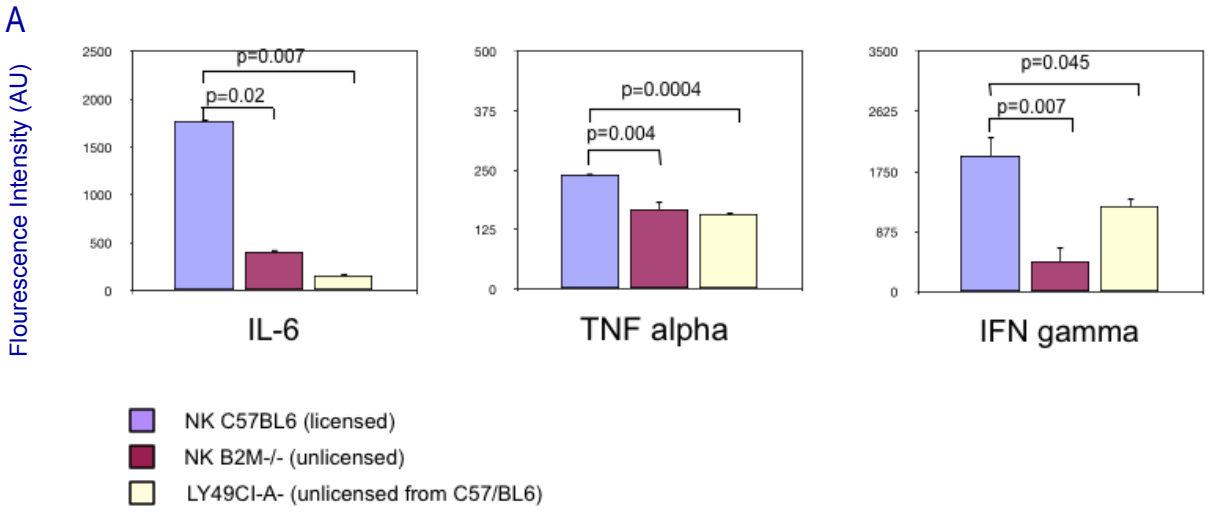

Figure 3-3. C57BL6 licensed NK cells produced higher amount IL-6, TNF-α, and IFN-γ.

Multiplex cytokine analysis of media from bulk NK cells of C57BL6 mice which include licensed NK cells have overall higher production of IL-6, TNF-α, and IFN-γ compared to unlicensed NK cells from $\beta 2m^{-/-}$ mice (which do not express HLA ligands and therefore lack the capacity to license NK cells). n = 2 to 3, Student's t-test, two-tailed.

Figure 3-4

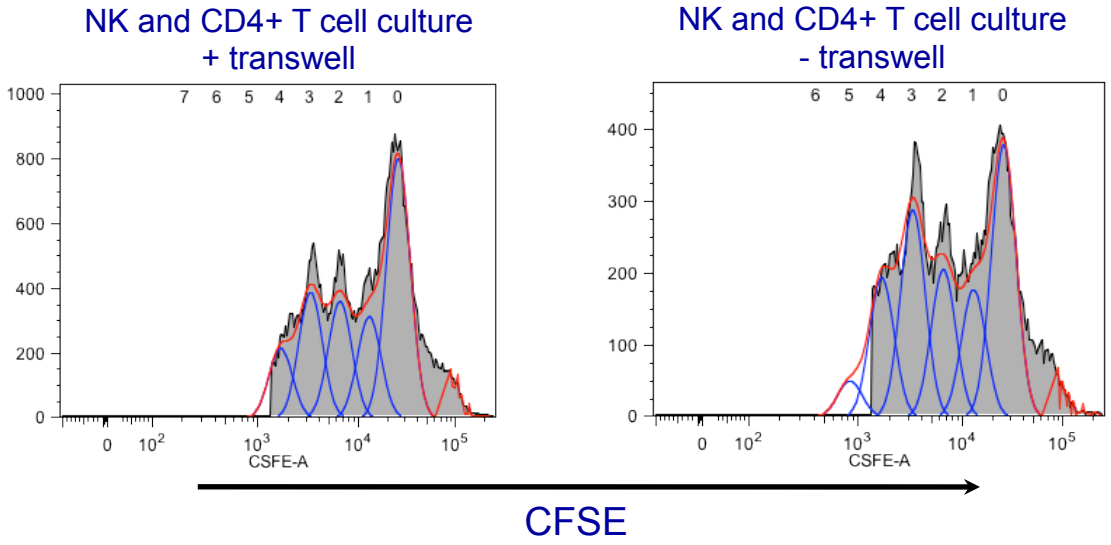

Figure 3-4. NK cells from C57BL6 mice co-activate $CD4^+$ T cell proliferation partially via contact-dependent mechanism.

Histogram of $CD4^+$ T cell CFSE dilution with and without separation between NK and T cells by 1.0 μm pore size transwell at NK:T ratio 1:1.

Figure 3-5

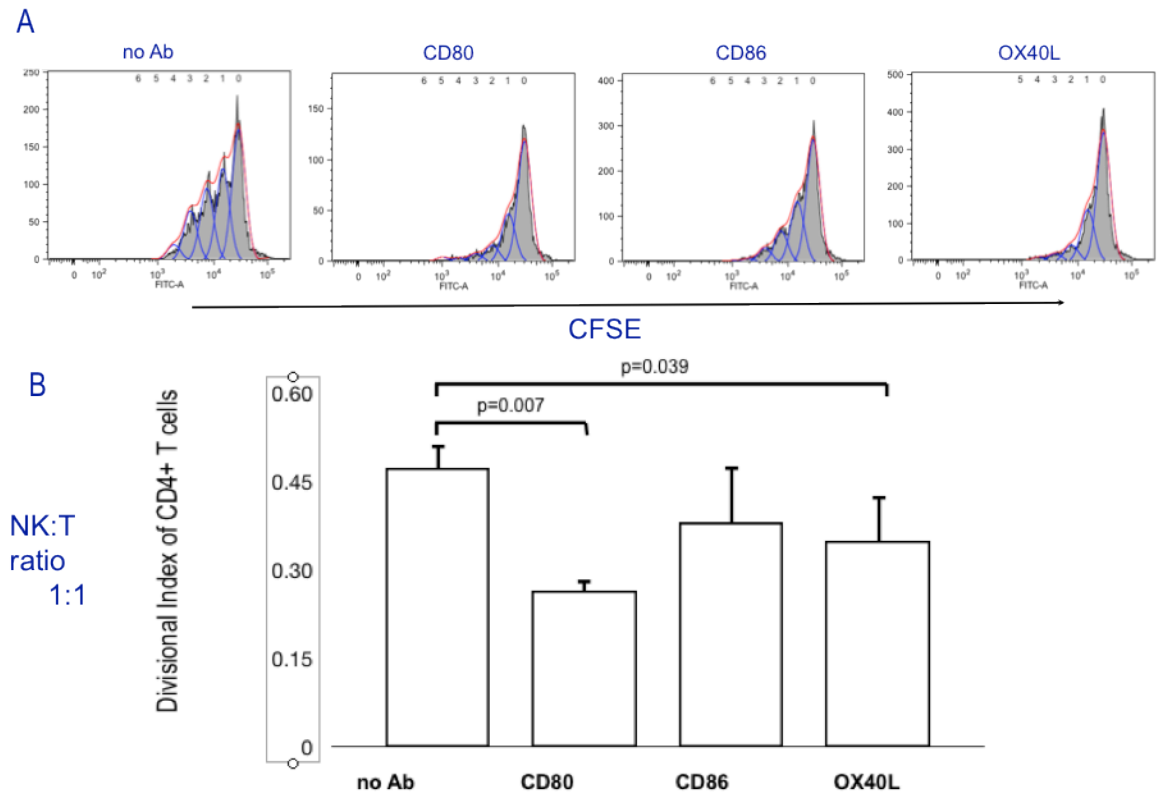

Figure 3-5. Blockade of candidate receptor-ligand interactions between NK and CD4$^+$ T cells diminished the impact of licensed NK cells.

(A) Addition of CD80 (B7-1), CD86 (B7-2), and OX40L antibody at 4ug/mL to NK:T co-cultures at ratio 1:1 resulted in decreased divisional index of CD4$^+$ T cell proliferation. (B) Histogram of CD4$^+$ T cell CFSE dilution in NK-CD4$^+$ T cell co-culture with and without addition of antibodies to CD80 (B7-1), CD86 (B7-2) and OX40L. n = 2, Student's t-test, two-tailed. Figure 3-6

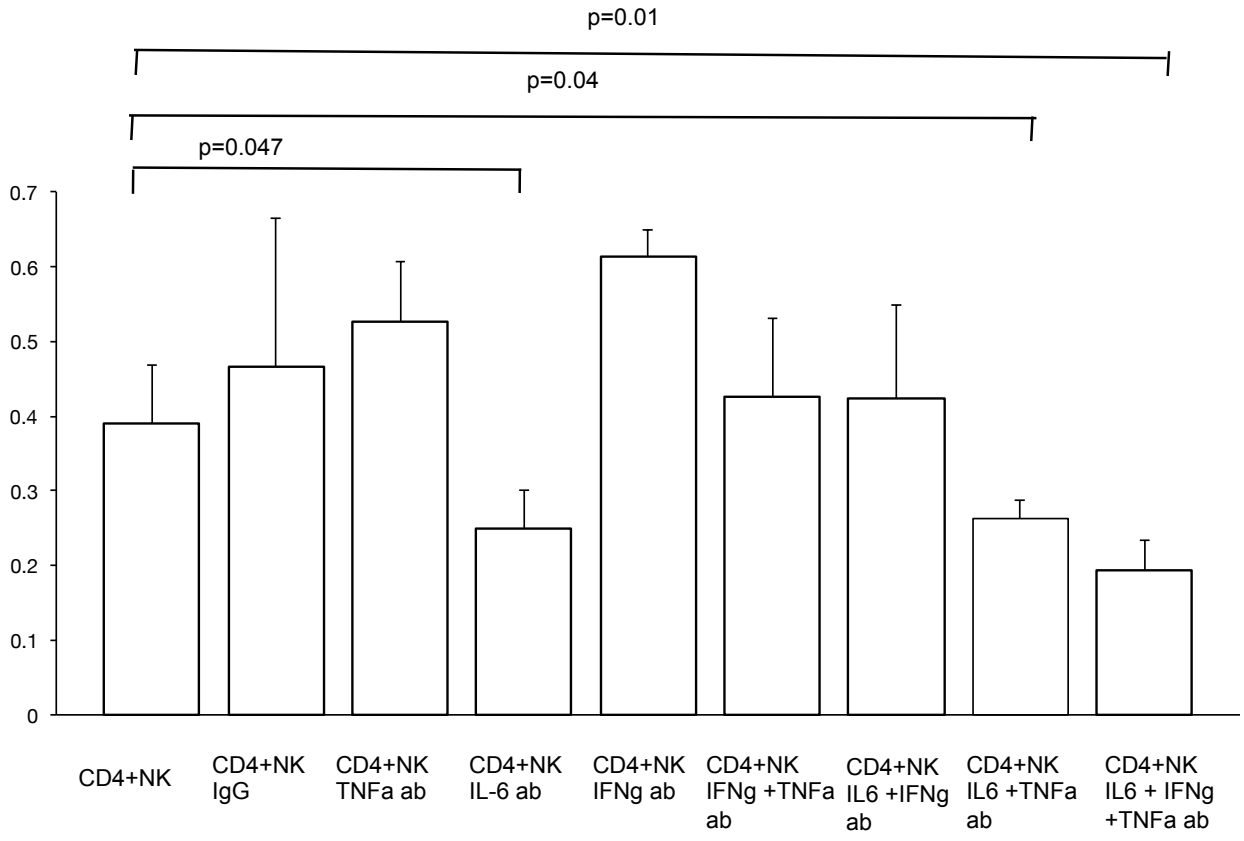

Figure 3-6. Blockade of candidate cytokines secreted by licensed NK cells diminish the proliferation of $CD4^+$ T cells.

(A) Neutralization of IFN-γ, TNF-α, IL-6, and their various combinations using neutralizing antibodies at 5μg mL^{-1} resulted in decreased divisional index of $CD4^+$ T cell proliferation. n = 2, Student's t-test, two-tailed.

Figure 3-7

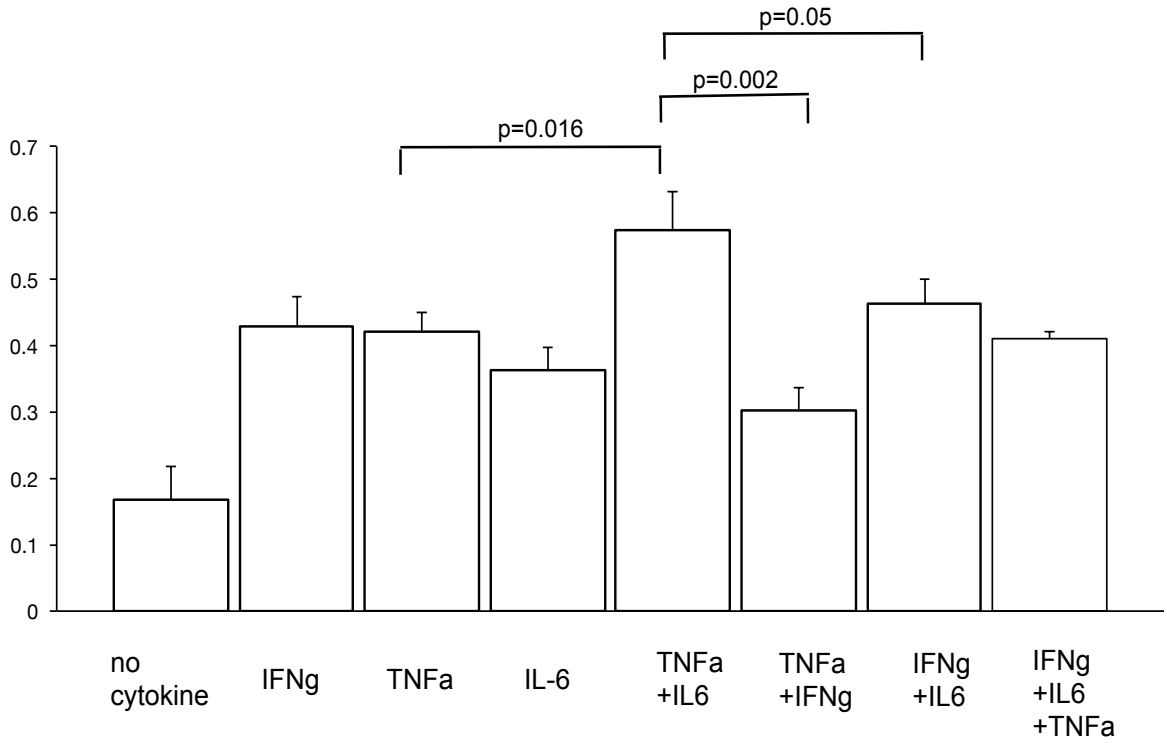

Figure 3-7. Addition of selected licensed NK cytokines promoted $CD4^+$ T cell proliferation.

Addition of IFN-γ, TNF-α, and IL-6 at 20 ng mL^{-1} to $CD4^+$ T cell 72-hour culture resulted in increased divisional index of $CD4^+$ T cell proliferation. n = 2, Student's t-test, two-tailed, (*p<0.05).

Figure 3-8

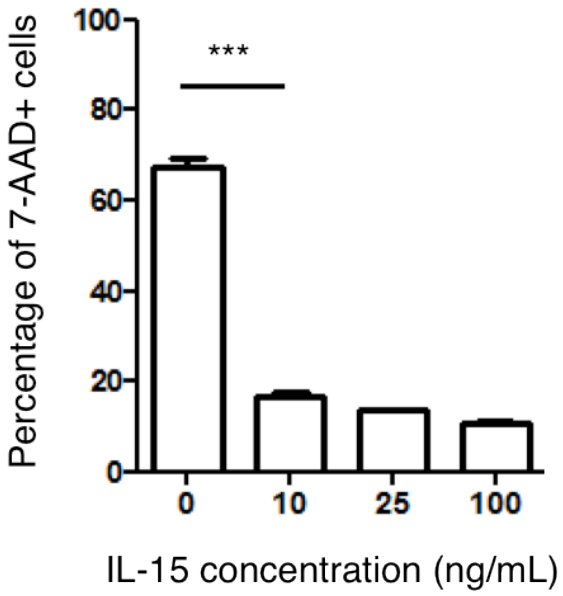

Figure 3-8. Brief stimulation with low dose IL-15 maintains NK cell viability *in vitro*.

IL-15 was added to NK cell culture at the dose of 10, 25, or 100 ng mL^{-1} for two hours in 37°C, and on ice for four hours to mimic sorting conditions. NK cells were then cultured for 96 hours. Brief stimulation with low dose IL-15 resulted in an increased survival rate (lower 7-AAD positive percentage) of NK cells at day 4. n = 4, Student's t-test, two-tailed, (***$p < 0.0001$).

Figure 3-9

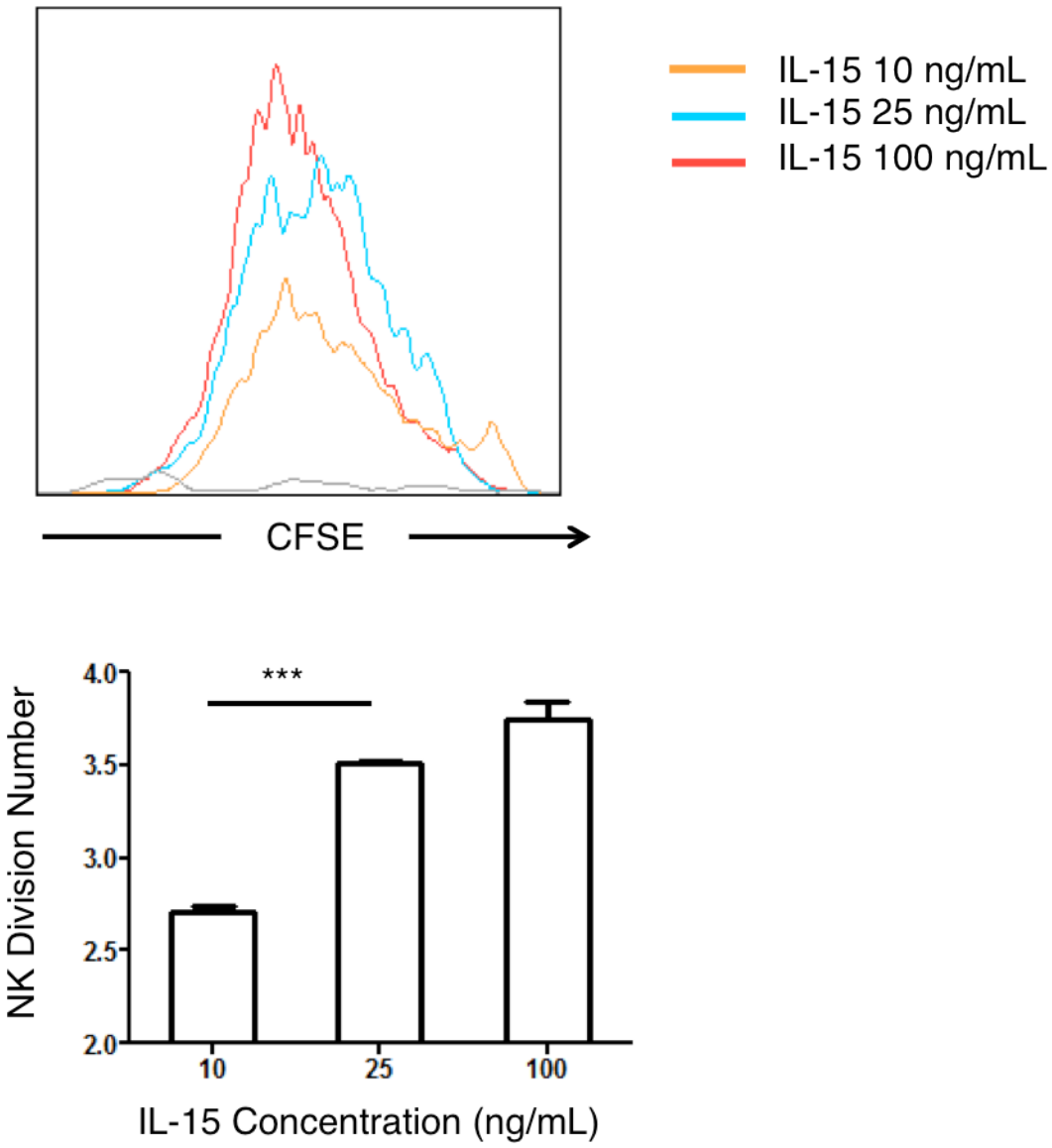

Figure 3-9. Brief stimulation with low dose IL-15 promotes NK cell proliferation *in vitro*.

IL-15 was added to NK cell culture at the dose of 10, 25, or 100 ng mL^{-1} for two hours in 37°C, and on ice for four hours to mimic sorting conditions. (A) NK cell proliferation after 96 hours is shown by the overlay of CFSE titration histograms. Orange: IL-15 10 ng mL^{-}

^{1}; Blue, IL-15 25 ng mL^{-1}; Red, IL-15 100 ng mL^{-1}. (B) Bar plot of the divisional index of NK cell proliferation treated with different doses of IL-15. n = 4, Student's t-test, two-tailed, (***p < 0.0001).

Figure 3-10

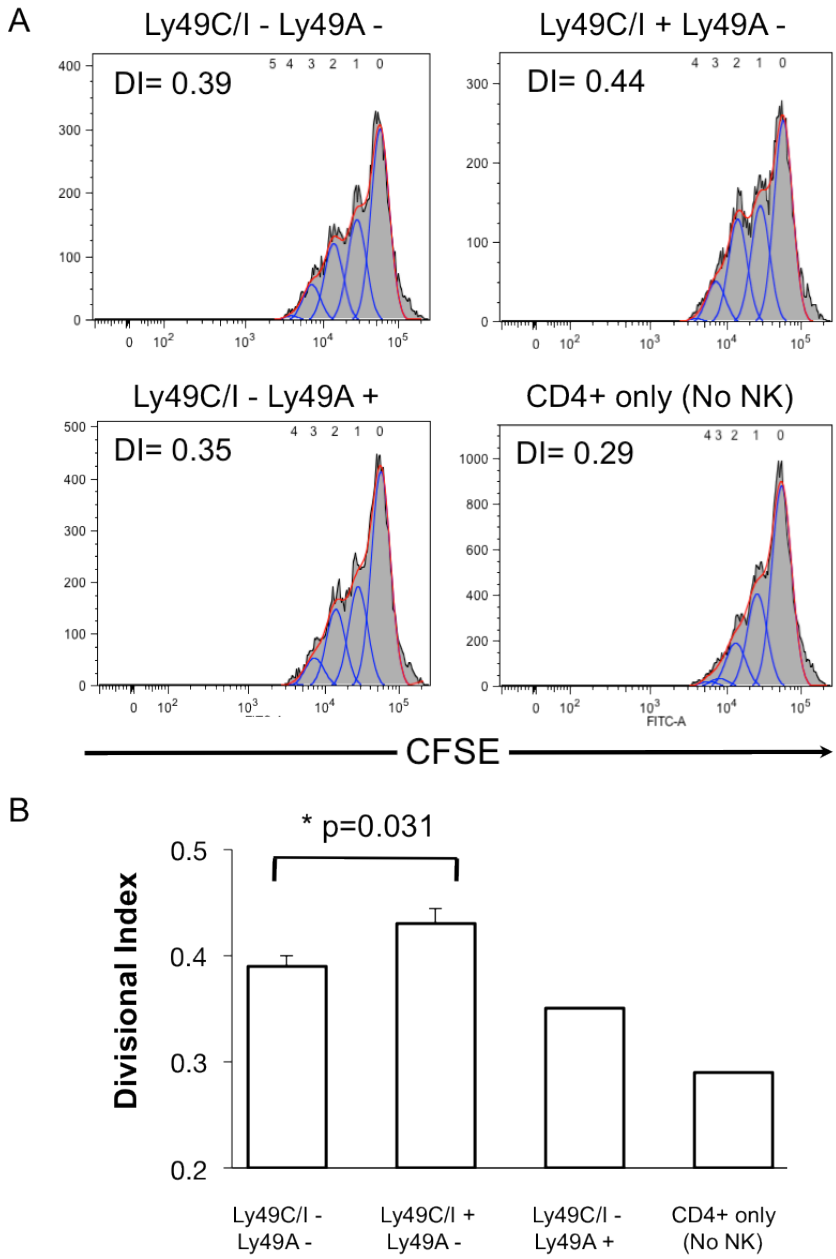

Figure 3-10. Sorted Ly49C/I^{+} Ly49A^{-} NK cells are more potent to facilitate CD4^{+} T cell proliferation compared to other subsets of NK cells.

IL-15 was added to PBMC cell culture at the dose of 10 ng mL^{-1} for two hours in 37°C, and sorted for Ly49C/I^{+} Ly49A^{-}, Ly49C/I^{-}Ly49A^{+}, and Ly49C/I^{-} Ly49A^{-} NK cells. (A) Histogram of CD4^{+} T cell proliferation after co-cultured with different subsets of NK cells

for 72 hours. (B) Barplot of the division index of $CD4^+$ T cell proliferation with different subsets of NK cells. n = 1-3, Student's t-test, two-tailed, (*$p<0.05$).

CHAPTER 4

Conclusions

Natural killer Cell Licensing: a Crohn's Disease Mechanism

Crohn's disease is a multi-factorial chronic inflammatory disease, with killer cell immunoglobulin-like receptors (KIRs) and their respective HLA class I ligands being identified as risk factors. However, the biologic basis of this genetic association is poorly understood. In this paper, we establish that, in normal and Crohn's disease patients, the impact of KIRs resides in the reprogramming of NK cells ("licensing") to induce a cytokine program which enhances $CD4^+$ T cell activation and T_H17 cell differentiation known to be critical in Crohn's disease pathogenesis.

NK licensing by KIR genes has been known for decades, but with this paper we uncovered the exceptional scope of biologic reprogramming achieved by NK licensing, its role in a common human disease, and the underlying mechanism (NK-mediated augmentation of adaptive T cell function). This observation is provocative in its genetics (a specific biologic mechanism accounting for a combinatorial genetic trait) and immunology (transforming the biologic understanding of NK licensing and its role in immune-mediated disease). Also, many chronic inflammatory, autoimmune, and infectious diseases have been associated with inhibitory KIR genes; this paper presents the first mechanistic explanation for this genetic association.

The fundamental mechanism by which NK licensing yields a distinct NK cell cytokine program is still emerging. In mice and humans, licensed NK cells gain higher sensitivity to cytokine stimulation,[24,27,73] but how the intracellular signaling network is rewired after NK licensing is unknown. Our inter- and intra-individual analysis of KIR-mediated licensing indicates that this rewiring affects a remarkably broad range of cytokines, which poses further challenges and conditions to the underlying mechanism

of NK licensing. Besides the exceptional breadth and pro-inflammatory cytokine profile of licensed NK cell, it is also conceivable that licensed NK cells, through their robust cytokine production, may contribute to the initiation of the pro-inflammatory process prior to the innate or immune activation of dendritic cells and macrophages. [27,40]

Finally, as the pathogenesis of Crohn's disease is ultimately driven by intestinal lymphocytes; it is desirable to confirm these properties of licensed NK cells resident in the gastrointestinal tract. However, due to the substantial technical difficulties in obtaining useful numbers of intraepithelial (IE) or lamina propria (LP) NK cells for functional studies, we are unable to accomplish this aim within the scope of the present study.

The aforementioned topics are essential to establish a firm foundation for the understanding of NK licensing in disease pathogenesis, and should be pursued with combined effort in the scientific community.

Single Cell Barcode Chip: a Technologic Novelty

NK cells are not abundant and are functionally heterogeneous, which poses significant challenges to understand individual cell behavior. We improved and utilized 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 time, allows us to identify the striking distinctions between licensed and unlicensed NK cell subsets, and the commonality within each subset.

Thiopurine Responsiveness Prediction: the Translational Significance

Certain anti-metabolite and biologic agents inhibit the function of lymphocytes, including NK cells. However, while such agents are widely used in chronic inflammatory disease, only some patients respond profoundly and durably. An implication of this study is that such agents may selectively benefit patients who genetically bear licensed NK cells. Thiopurines drugs are one group of such agents. They are a potent class of rac1 inhibitors that functionally attenuate several classes of lymphocytes, and are widely used for Crohn's disease and other autoimmune disease treatment. However, thiopurines have toxicity and only certain patients respond to them. As a pharmacogenomic corollary, we show that clinical responsiveness to thiopurine is dependent on the KIR/HLA genetics. This suggests that NK cells are key clinical targets of thiopurines, and suggest that licensing genetics can be applied to identify individuals are most likely to benefit from this therapy. Our next step is to validate this clinical finding using an independent and bigger Crohn's Disease cohort.

NK Licensing in Murine Models: Validation of the Mechanism

In humans, evidence suggests that genetic licensing is a risk factor for Crohn's disease and recent work (L Lin et al) uncovered diverse cytokine production programmed by genetic licensing. However, limited and discrepant studies exist on the relationship of NK cells to immune mediated colitis. To biologically test this genetic association, I also have been investigating the interaction between murine licensed NK cells and $CD4^+$ T cells which are well known mediators of inflammation in mouse colitis

models. Our results demonstrate that licensed murine NK cells augment $CD4^+$ T cell proliferation and this may be mediated by increased secretion of proinflammatory mediators such as IL-6 and TNF-α, as well as cell-to-cell contact dependent mechanism. This feature of murine licensed NK cell is different from the solely cytokine-mediated mechanism seen in human licensed NK cells.

One major aim of this study is to model the role of NK cell licensing in immune-mediated colitis. To investigate this, we will introduce sorted licensed NK cell subsets into $il2rg^{-/-}rag2^{-/-}$ recipient mice that have $CD4^+CD45RB^{hi}$ T cell-induced colitis. Standard methodologies, such as body weight monitoring, histology scoring, and immunological trait characterization will be employed to assess the correlation of NK licensing with colitis development and pathogenesis.

SUPPLEMENTAL CHAPTER 1

Intestinal Microbes Affect Phenotypes and Functions of Invariant Natural Killer T Cells in Mice

Abstract

Background & Aims

Invariant natural killer T (iNKT) cells are a subset of T cells expressing invariant T-cell antigen receptor (TCR). Their TCRs recognize glycolipids presented by CD1d, a nonpolymorphic major histocompatibility complex (MHC) class I-like antigen-presenting molecule. iNKT cells have been implicated in a wide variety of inflammatory diseases. Their memory phenotype and rapid effector functions to antigenic stimulation suggested possible previous exposure to antigens. However, little is known about this process. The overall aim of this paper is to investigate whether commensal microbes determine the activation and function of iNKT cells. The aim of my contribution to this paper is to determine if a limited set of intestinal microorganisms would affect the phenotype and responsiveness of iNKT cells.

Methods

We compared the phenotype and responsiveness of iNKT cells from mice carrying different intestinal microbiota from housing in different environments.

Results

iNKT cells derived from mice carrying altered microflora displayed higher usage of $V\beta7^{+}$ in spleen. These iNKT cells have a reduced activation level and reduced responsiveness to antigenic stimulation.

Conclusion

Intestinal microbes can affect murine iNKT cell phenotype and responsiveness to antigenic stimulation.

Introduction

Invariant NKT cells are a subset of T lymphocytes characterized by the expression of an invariant TCR that undergoes canonical Vα14-Jα18 rearrangement in mice. This set of TCR recognizes antigens presented by CD1d, a nonpolymorphic MHC class I-like molecule.[90-92] CD1d binds lipid structures, and one of the most well-known ligands is αGalCer, a synthetic glycolipid ligand.[90]

iNKT cells have been found to mediate the pathogenesis of many inflammatory conditions of the alimentary tract, including liver, pancreas, and intestines. Although human data have been sparse, available data suggested a comparable role of human iNKT cells in different disease contexts. iNKT cells can be either protective or detrimental in different disease models, depending whether they are more polarized towards Th1 or Th2 direction.[90-92] In the case of inflammatory bowel disease, it is consistently shown that iNKT cells are beneficial in Th1 mediated processes, while deleterious in Th2 mediated processes.[93,94] iNKT cells appear constitutively active and express surfaces markers indicative of memory phenotypes. Upon antigen stimulation, they initiate rapid effector functions, including production of Th1 and Th2 type cytokines and cytotoxicity. [90-92]

The memory phenotype and rapid effector functions of iNKT cells indicate they might have early antigen encounters; however this process is largely unclear. Glycolipid antigens from a few types of bacteria have been reported to stimulate the majority of murine iNKT cells.[95-99] Among them, the Sphingonomas/Sphingobium species have glycosphingolipids similar to αGalCer.[97,98] Other commensal and pathogenic bacteria species have also been reported to activate iNKT cells. However, how murine intestinal

microbiota and the wider environment shape the phenotype and functions of iNKTs in non-inflammatory conditions remain to be investigated.

Materials and Methods

Mice

Mice were housed under specific pathogen-free (SPF) conditions at the animal facilities of the UCLA Division of Laboratory Animal Medicine (Los Angeles, CA), in accordance with the Institutional Animal Care Committee (ARC) guidelines. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). SPF C57BL/6 mice bear normal aerobic and anaerobic enteric commensal microflora are negative for a panel of pathogenic microorganisms based on serologic and microbiologic screening by the UCLA Division of Laboratory Animal Medicine. A restricted flora (RF) colony was established by extensive antibiotic treatment and recolonization with six species of nonpathogenic *Clostridium.*[100,101 102] For both SPF and RF mice, animals of either gender were used at age 6 to 12 weeks.

Reagents and monoclonal antibodies

αGalCer ((2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol) was provided by Kronenberg lab (La Jolla Institute for Allergy and Immunology) obtained from the Kirin Pharmaceutical Research Corporation (Gunma, Japan). αGalCer-loaded CD1d tetramers were produced and provided by Kronenberg lab. [101,103] Antibodies against mouse TCRβ7, NK1.1, CD69, IFN-γ, TNF-α, IL-4 were purchased from BD Biosciences (San Diego, CA).

Cell Preparation, In Vivo Challenge, and Flow Cytometry

Single-cell suspensions from spleen, were prepared as described.[87,88] Briefly, spleens were smashed and filtered through 70 micro strainer to extract splenocytes. RBCs were then lysed (ACK Lysis buffer, Lonza, Rockland, ME) and white blood cells (WBC)s were washed once with medium (RPMI 1640; Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) FBS (Mediatech, Manassas, VA), 1% (v/v) penicillin-streptomycin-glutamine (10,000 U/ml penicillin, 10,000 µg/ml streptomycin, 29.2 mg/ml L-glutamine; Invitrogen), and 50 µM β-mercaptoethanol (Sigma-Aldrich). Cell staining for flow cytometry was performed as reported previously.[88] iNKT cells were activated *in vivo* by intravenous injection of 1 µg αGalCer and analyzed 90 minutes later.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Comparisons were drawn using a 2-tailed Student's t-test or an analysis of variance test. P values < .05 were considered significant and are indicated as follows: *P < .05, **P < .01, and ***P < .001. Each experiment was repeated.

Results

Restricted murine microbiota affects the activation and response of iNKT cells

To analyze the effect of a limited set of intestinal organisms on the phenotype and responsiveness of iNKT cells, here we compared the iNKT cells between (restricted flora) RF and (specific pathogen free) SPF mice. RF mice carry an altered and reduced species of intestinal microorganisms, including both fungal and bacterial species.[100 104] Compared to SPF mice, RF mice lack Sphingomonas/Sphingobium species, which are known to activate iNKT cells, and is enriched for Firmicutes specie. [105] lthough the cell numbers of iNKT are reduced in RF mice, their phenotype, activation state, and cytokine production in response to αGalCer can still be assessed. Splenic iNKT cells derived from RF mice displayed a higher frequency of $V\beta7^+$ usage (Figure S1-2). Under resting conditions, splenic iNKT cells from RF mice expressed lower levels of CD69 compared to SPF mice. Although splenic iNKT cells derived from both RF and SPF mice respond to αGalCer stimulation based on their CD69 expression, the degree of CD69 up-regulation is less in RF mice (Figure S1-1). Furthermore, in response to αGalCer stimulation, there were less iNKT cells from the RF mice that were capable of producing cytokines, including IFN-γ, TNF-α, and IL-4 (Figure S1-1). Our results recapitulated the data we obtained for the other parts of the paper.[101]

Discussion

Invariant NKT cells are characterized by their expression of invariant T-cell receptor, which recognizes glycolipid antigens presented by the non-polymorphic MHC class I molecule CD1d. iNKT cells play important roles in multiple immunological diseases, either beneficial or detrimental, depending on their polarization in the disease context. iNKT cells have effector memory like phenotype, and respond to antigenic stimulate rapidly, suggesting probable antigen encounters earlier in their life. This paper demonstrated that the gut microbiota is a key component of the functional education program that affects the phenotypical and functional outcomes of iNKT cells. The lack of Sphingomonas/Sphingobium species in RF mice explained the reduced levels of activation and responsiveness of iNKT cells derived from RF mice, and is consistent with the other parts of the story presented in the paper. At the same time, it is unlikely that one single species would be responsible for all the differences; it is worth pursuing to identify other associated microorganisms, including both bacterial and fungal species.

Figures and Tables

Figure S1-1

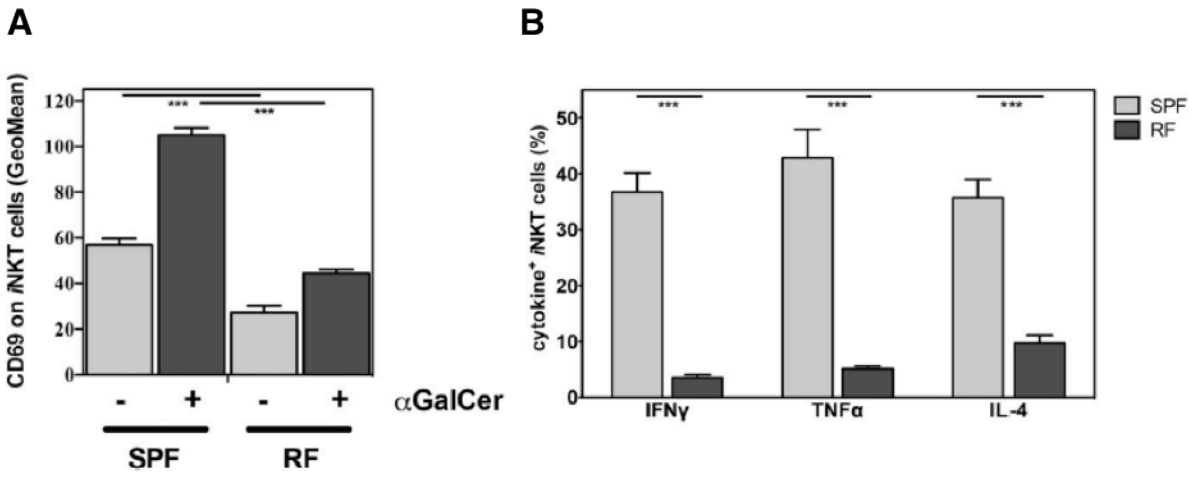

Figure S1-1. Bacterial exposure corrects the hyporesponsive phenotype of iNKT cells.

(A) Expression of CD69 and (B) indicated cytokines by splenic **i**NKT cells from RF and SPF-housed C57BL/6 mice with or without αGalCer challenge *in vivo* (90 min). Representative data from two independent experiments are shown. ***P < .001.

Figure S1-2

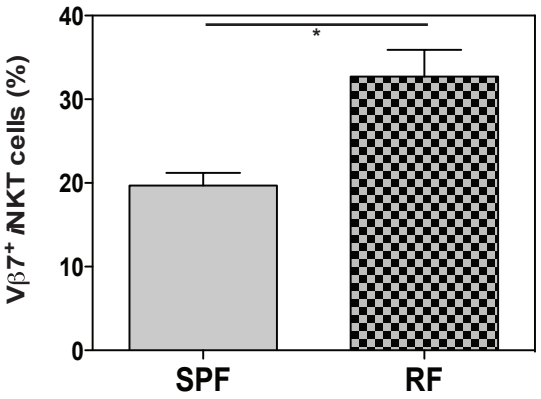

Figure S1-2. Frequency of Vβ7+ iNKT cells in RF mice.

Splenic iNKT cells from restricted flora (RF) or specific pathogen free (SPF) housed C57BL/6 mice for the frequency of Vβ7. p = 0.022. The graph summarizes data from two independent experiments, with 3 mice per group.

SUPPLEMENTAL CHAPTER 2

Innate Lymphoid Cell Function and Mucosal Ecosystem in Defective Barrier-induced Murine Colitis

Abstract

Background & Aim

Ulcerative colitis (UC) is an intestinal immune disorder that manifests in the distal colon. Abnormal glycosylation of mucin proteins, the basic components of mucus layer, has been associated with pathogenesis of UC. Mice with defective mucus production by intestinal epithelial cells (IEC C1galt1$^{-/-}$) have mucosal barrier dysfunction and develop spontaneous innate colitis. ILC are a distinct family of innate lymphocytes that mediate mucosal homeostasis, therefore, our aims are to 1) investigate the roles of ILCs in colitis induced by defective mucus barrier function; 2) to investigate how defective intestinal barrier function changes gut ecosystem, including microbiota , metabolomics, and proteomics.

Methods

Tissues, microbial DNAs, and lavage samples from different anatomical regions of intestines from IEC C1galt1$^{-/-}$ mice at different ages were collected. Phenotyping and functional assessment of different subsets of ILCs from various locations of the intestine will be performed using flow cytometry. Microbial 16S DNA will be extracted and sequenced by Illumina; colonic lavage samples will be collected for metabolomics and proteomics analysis through collaboration with experts in the field.

Results

IEC C1galt1$^{-/-}$ conditional and IEC C1galt1$^{-/-}$ Rag$^{-/-}$ conditional knockout mice strains have been re-derived into UCLA animal facilities and bred. Immune phenotypes and cytokine production functions of ILC subsets from multiple intestinal regions of IEC C1galt1$^{-/-}$ mice have been assessed. Since my major thesis project focuses on human

NK cells, aim 2 was taken over by a coworker in the lab. Besides the effort with the mouse strains, I also co-processed and collected tissue samples, luminal contents, and lavage samples for microbiome, metabolomics, proteomics and immunologic studies.

Conclusion

This project is still in progress; preliminary results will need to be validated to draw any conclusion.

Introduction

Mucins and O-glycan

Mucus layers play important roles in preventing intestinal microbial antigens invading the mucosal immune system.[106,107] Colon mucus contains two layers: a loose outside layer and a dense inside layer. The outside layer hosts commensal bacteria; whereas the inside layer is largely bacteria-free and separates the epithelium from the commensal microfloral.[108] The mucus layer is made up of polymerized mucins produced by goblet cells.[107,109] Mucins are heavily glycosylated proteins that carry large numbers of O-linked oligosaccharides (O-glycans).[108,110,111] All O-glycans started off from a primary structure called Tn antigen (GalNAcα-O-Ser/Thr; Figure S2-1). Tn antigens are normally glycosylated to form core 1-derived structures, the main type of O-glycan,[111] thus they normal remain unexposed. The biosynthesis of core 1-derived structures is mediated by core 1 β1, 3-galactosyltransferase (C1galt1, also known as T-synthase) and the specific ER-resident chaperone, C1GALT1C1, for C1galt1, therefore, a defective expression of C1galt1 or C1GALT1C1 will cause the exposure of Tn antigen.[111-113]

Ulcerative Colitis and Mucins

Ulcerative colitis (UC) is an intestinal immune disorder that manifests in the distal colon.[114] It has been observed that UC patients have altered mucus layer, abnormal mucin expression, and aberrant O-glycosylation in the distal colon.[115-119] Approximately 30% of UC patient colon biopsies have been detected of Tn antigen exposure, indicating the presence of defects in the core 1 or core 3 O-glycosylation pathways.

[111,112] Since the expression levels of core 3 β1,3-N-acetylglucosaminyltransferase in UC and normal control colons are comparable, the defects in pathways controlling core 1 O-glycosylation is likely to be accountable for the exposure of Tn antigen.[120] Somatic mutations in X-linked *C1GALT1C1*, essential for normal core 1 O-glycosylation processes, have been detected, including 4 missense loss-of-function mutations in the coding sequence of *C1GALT1C1*, in UC patients with aberrant O-glycosylation.[120-122]

IEC C1galt1$^{-/-}$ mice develop spontaneous colitis

Core 1 derived O-glycans are expressed in many tissues, including intestinal epithelium. A mouse model with targeted deletion of C1galt1 in intestinal epithelial cells (IEC C1galt1$^{-/-}$, or simplified as Tsyn$^{-/-}$) was generated by crossing mice with loxP sites flanking C1galt1 (C1galt1 $^{f/f}$)[123] with an intestinal epithelium-specific Cre-expressing transgenic line (VillinCre mice). [120,124] This strain of mouse develops spontaneous colitis that resembles human UC, characterized by infiltration of massive TNF-α-producing myeloid cells and presence of crypt abscesses. Mechanistic studies suggested that innate rather than adaptive immune cells are mediating the inflammatory processes. [109]

Innate lymphoid cells (ILCs)

ILC are a distinct family of lymphocytes from B or T cells, because their developmental pathway is unique and they are equipped with environmental sensors rather than antigen-specific receptors. Localized to the mucosa and related stromal tissues, ILCs bear a cellular physiology designed for rapid, innate protective responses.[1] ILC subsets parallel the activity of T cell subsets and are involved in

responses to all classes of pathogens. The first identified ILC was the natural killer (NK) cells, which produce Th1 cytokines, and hence have been termed ILC1. Type 2 ILC (ILC2) is defined by its production IL-5 and IL-13, and plays important roles in early eosinophil recruitment and mucosal processes for worm expulsion in helminth infection.[20,21] Type 3 ILCs (ILC3) includes lymphoid tissue inducers (LTi), IL-22-producing ILC (ILC22) and IL-17-producing ILC (ILC17). LTi cells are critical organizers of lymphoid tissue formation and adaptive immunity; upon mucosal bacterial infection and attendant mucosal myeloid IL-23, LTi cells are the major early producers of IL-17 and IL-22, and hence important for protective neutrophil recruitment (IL-17) and epithelial defense and survival mechanisms (IL-22).[15,16] ILC22 is present in intestine, mainly residing in small intestine lamina propria. Their development requires IL2 and IL-15 signaling,[18] as well as microbial flora for differentiation. ILC17 accumulates at sites of inflammation in experimental microbial and immune-mediated colitis, and infection associated IL-23 production stimulates ILC17 to produce large amount of IL-17 and IFN-γ.

Microbiota Gardening

IBD is a multi-factorial disease that results from the interaction of environmental cues, including intestinal microbiota with host immune regulation in genetically susceptible individuals[125]. Observations in patients with IBD and in animal models have clearly demonstrated that intestinal microbiota dysbiosis or perturbation is associated with the onset and perpetuation of inflammation[126]. Host microbiota is consistently being shaped, including exposure to colonizers and pathogens, diet, and host immune selection.[127] The mucosal barrier and immune system actively “garden” the microbiota

to maintain a beneficial flora. Putative gardening cell types include Paneth cells by secreting defensins and RegIIIg, epithelial cells through inflammasome, NKT cells through CD1d, et al.[128] Here our aim is to investigate how defective intestinal barrier changes gut ecosystem, including microbiota , metabolomics, and proteomics.

Materials and Methods

Mice breeding and genotyping

The animal studies were conducted with protocols that had been approved by the Animal Research Committee of UCLA. Mice were raised in a specific pathogen–free barrier facility at UCLA Division of Laboratory Animal Medicine, and genotyped routinely by Transnetyx (Cordova, TN) using genomic DNA isolated from tail or ear clips and. The primers used by Transnetyx for genotyping were selected from their database not publicly disclosed. The breeding strategies for IEC C1galt1$^{-/-}$ conditional knockout colony and C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag1$^{-/-}$ conditional knockout colony were explained in detailed in the results section.

Splenocytes and intestinal lymphocytes isolation

Single-cell suspensions from spleen, distal colon, proximal colon, cecum, ileum, mid small intestine, proximal small intestine, Peyer's patch, and mesenteric lymph nodes (MLN) were prepared as described previous.[129] Briefly, for spleen, tissue from individual mice was homogenized gently in a 100-mm cell strainer in a petri dish containing DMEM with antibiotics and 10% FCS (Life Technologies, Long Island, NY). Splenocytes were recovered by centrifugation at 2000 rpm for 5 minutes; RBCs were lysed with ACK lysis buffer (Lonza, Walkersville, MD). SI and LI IELs were isolated as described, [87] with minor modification. Briefly, MLN were taken out before dissecting intestinal tissues. SI and LI were cut open longitudinally, removed of Peyer's Patches, and washed extensively with DMEM media to remove lumen contents. MLN and Payer's Patch single cell suspensions were obtained by homogenizing MLNs gently in a

100 μm cell strainer, and collected by centrifugation. Intestinal tissues were then cut into 5-mm pieces and incubated with for 30 min at 37°C for DDT digestion (15 ml DMEM media containing 10% FCS and 1 mM DTT). Supernatants were filtered through 100 μm cell strainer (BD); cell pellets were resuspended in 40% Percoll DMEM media containing and 10% FCS, layered over 80% Percoll DMEM media, and intraepithelial lymphoctyes (IEL) were collected as the cell at Percoll gradient interface. The residual tissue pellets were further digested by collagenase and DNAase (15mL DMEM media containing 10% FCS, 0.5mg mL^{-1} collagenase and 0.5mg/ml DNase I) for 30 minutes at 37°C in shaker at 150 rpm. The supernatant were filtered and interface cells were collected after Percoll gradient and used as lamina propria lymphocytes (LPL). All collected cells were expanded in 50ng mL^{-1} IL-23 DMEM media or DMEM media alone overnight before flow cytometry analysis.[101]

Antibodies

Surface markers anti-CD3, CD4, Thy-1, CD127, NKp44, NKp46, B220, and CD14 were purchased from eBioscience (San Diego, CA). PE- or APC- conjugated anti-IL-22, IL-17A, Rorgt, Foxp3, and recombinant murine IL-23 were purchased from eBioscience.

Microbial DNA preparation and sequencing

DNA was extracted from the mucosa of the distal colon, proximal colon, cecum, ileum, mid small intestine, and proximal small intestine. The intestines were first dissected, rinsed with medium to remove feces, incubated in DTT to release the

epithelium and adherent mucus (details described earlier). Colonic lavage specimens from the distal colon were also obtained to allow comparison of mucosa-associated bacteria and luminal bacteria. Microbial 16S DNA was amplified by PCR then sequenced using Illumina. Demultiplexed sequence data was run through the QIIME pipeline to select OUTs (Operational taxonomic units) *de novo*, remove singleton OTUs, and assign taxonomic identification to OTUs.

Results

Breeding IEC C1galt1$^{-/-}$ and C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag1$^{-/-}$ conditional knockout colonies

IEC C1galt1$^{-/-}$ colony was maintained by breeding C1galt1$^{f/f}$ IEC Cre$^{+/-}$ males with C1galt1$^{f/f}$ IEC Cre$^{-/-}$ females (Figure S2-2); the resultant C1galt1$^{f/f}$ IEC Cre$^{+/-}$ males and C1galt1$^{f/f}$ IEC Cre$^{-/-}$ females will be used as future breeders, while C1galt1$^{f/f}$ IEC Cre$^{+/-}$ with C1galt1$^{f/f}$ IEC Cre$^{-/-}$, as littermate controls, will be used for pilot experiments. This strain was successfully maintained, and we have frozen 10 straws 135 embryos down and stored in the UCLA animal facilities.

IEC C1galt1$^{-/-}$ Rag1$^{-/-}$ colony was bred by first mating C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag1$^{+/+}$ with C1galt1$^{-/-}$ IEC Cre$^{-/-}$ Rag1$^{-/-}$ to generate C1galt1$^{f/-}$ IEC Cre$^{+/-}$ Rag1$^{+/-}$ and C1galt1$^{f/-}$ IEC Cre$^{-/-}$ Rag1$^{+/-}$ progeny. The breeding scheme (Figure S2-3) using these progenies as parental breeders was employed to generate C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag1$^{-/-}$ males and C1galt1$^{f/f}$ IEC Cre$^{-/-}$ Rag1$^{-/-}$ females as future breeders; Ultimately, the C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag1$^{-/-}$ males were bred with C1galt1$^{f/f}$ IEC Cre$^{-/-}$ Rag1$^{-/-}$ females to maintain the colony as well as for direct experiments. This strain has been successfully bred, and we have frozen down 15 straws 232 embryos and stored in the UCLA animal facilities.

Figures and Tables

Figure S2-1

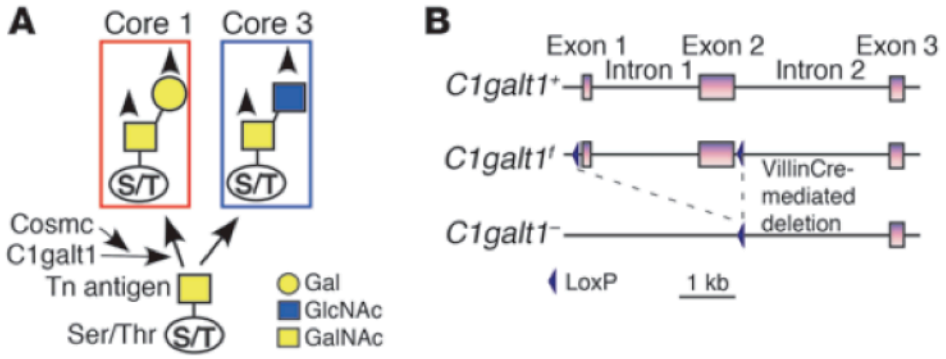

Figure S2-1. Generation of IEC C1galt1$^{-/-}$ mice

(A) Scheme for the biosynthesis of core 1 and core 3 O-glycans. Arrowheads indicate possible further branching to form core 1– or core 3–derived O-glycans, and possible fucosylation, sialylation, and sulfation. (B) Strategy for generating IEC C1galt1–/– mice. After crossing C1galt1$^{f/f}$ mice with VillinCre transgenic mice, VillinCre-mediated recombination deletes exons 1 and 2 of C1galt1 specifically in intestinal epithelium.

Figure S2-2

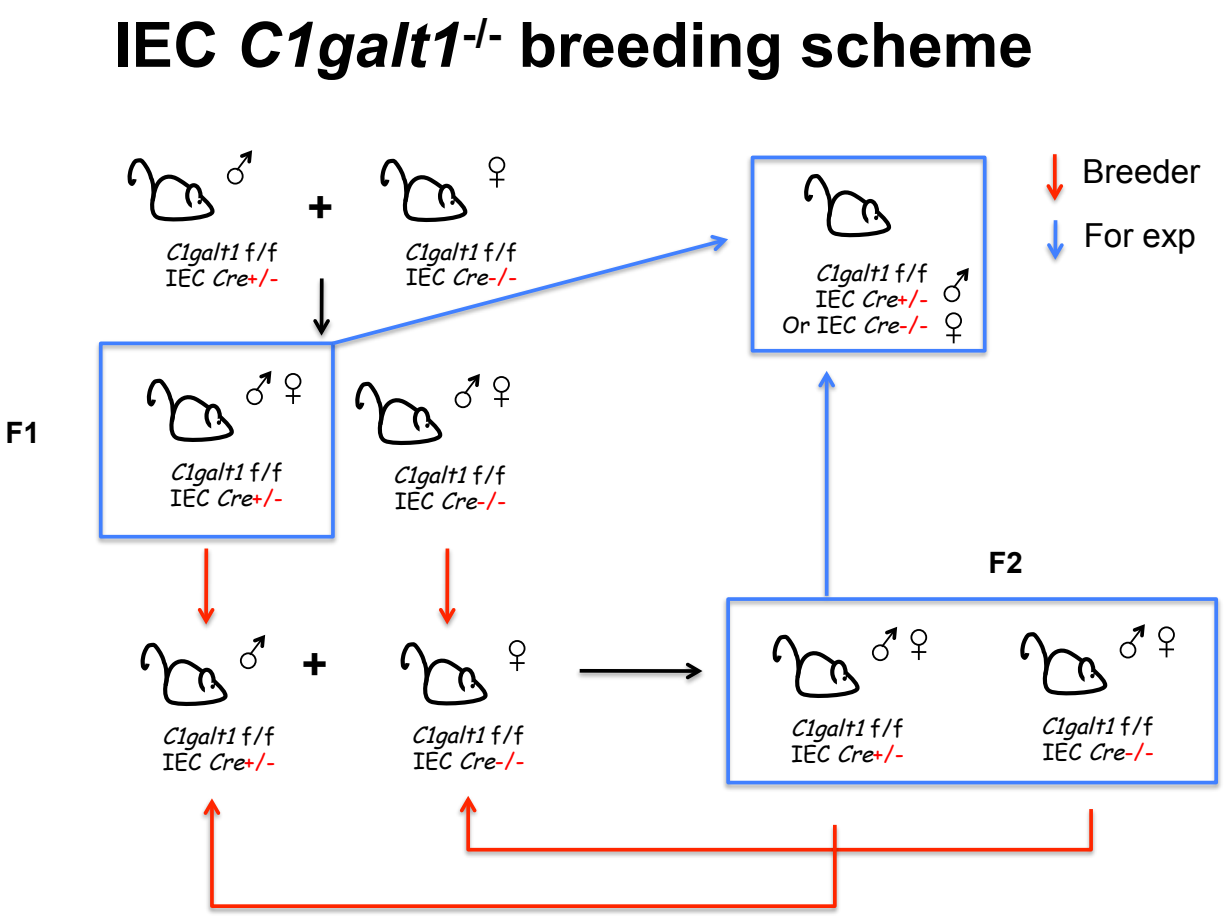

Figure S2-2. Breeding scheme for IEC C1galt1$^{-/-}$ conditional knockout mice

IEC C1galt1$^{-/-}$ colony was maintained by breeding C1galt1$^{f/f}$ IEC Cre$^{+/-}$ males with C1galt1$^{f/f}$ IEC Cre$^{-/-}$ females. Resultant C1galt1$^{f/f}$ IEC Cre$^{+/-}$ males and C1galt1$^{f/f}$ IEC Cre$^{-/-}$ females will be used for breeding, while C1galt1$^{f/f}$ IEC Cre$^{+/-}$ females with C1galt1$^{f/f}$ IEC Cre$^{-/-}$ males will be used for pilot experiments.

Figure S2-3

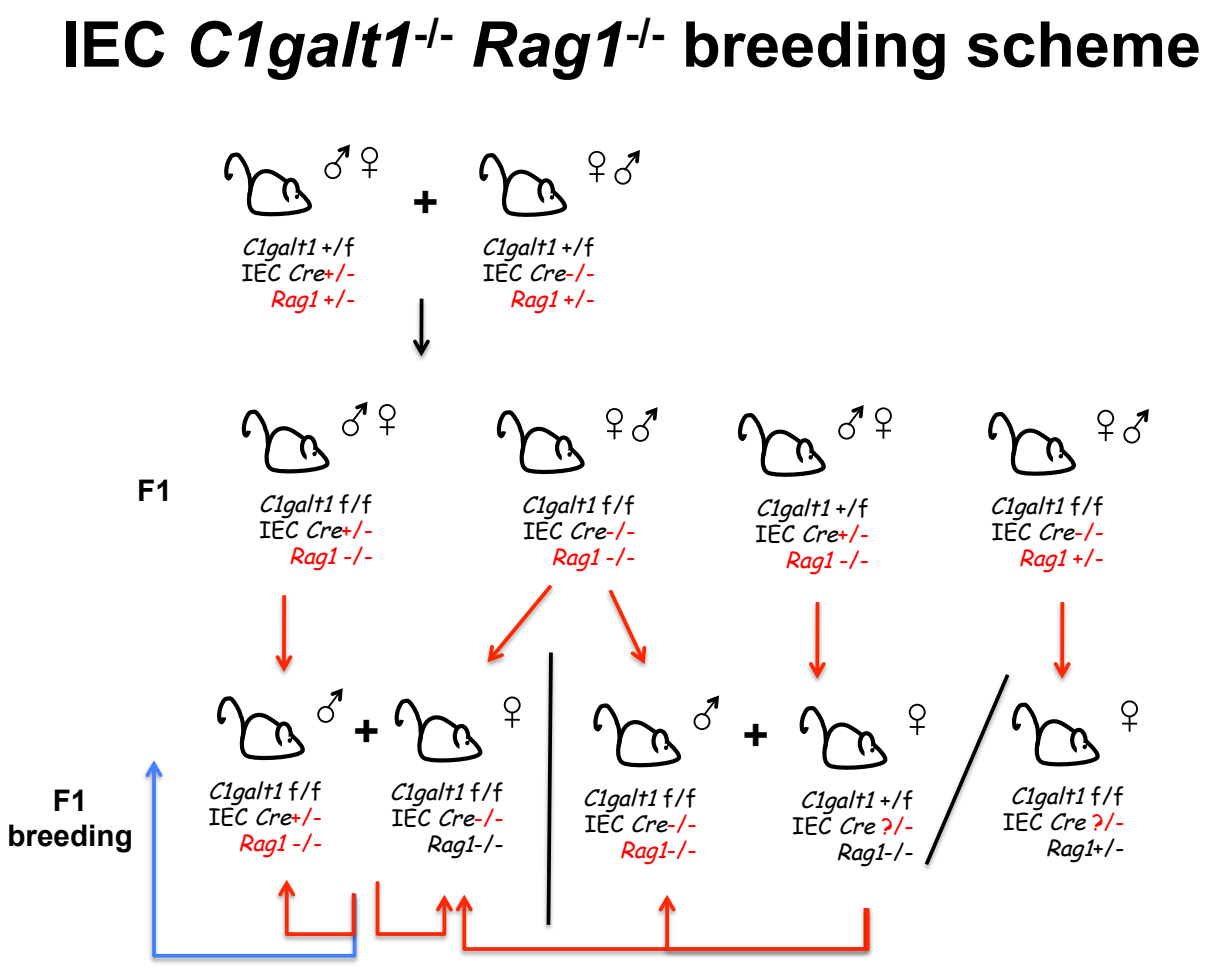

Figure S2-3. Breeding scheme for IEC C1galt1$^{-/-}$ Rag1$^{-/-}$ conditional knockout mice

IEC C1galt1$^{-/-}$ Rag1$^{-/-}$ colony was bred by first mating C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag1$^{+/+}$ with C1galt1$^{-/-}$ IEC Cre$^{-/-}$ Rag1$^{-/-}$ to generate C1galt1$^{f/-}$ IEC Cre$^{+/-}$ Rag1$^{+/-}$ and C1galt1$^{f/-}$ IEC Cre$^{-/-}$ Rag1$^{+/-}$ males and females, which serve as the breeder in the above scheme. First, C1galt1$^{f/-}$ IEC Cre$^{+/-}$ Rag1$^{+/-}$ and C1galt1$^{f/-}$ IEC Cre$^{-/-}$ Rag1$^{+/-}$ were mated to generate F1 progeny. F1 pups were genotyped and selected for genotypes of C1galt1$^{f/f}$ Rag1$^{-/-}$, C1galt1$^{f/f}$ Rag1$^{+/-}$, or C1galt1$^{f/-}$ Rag1$^{+/-}$ as breeders (Cre locus were not considered at this step). Resultant C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag1$^{-/-}$ males were then bred with C1galt1$^{f/f}$ IEC Cre$^{-/-}$ Rag1$^{-/-}$ females; all their pups could be directly used as future

breeders or for direct experiments (C1galt1$^{f/f}$ IEC Cre$^{-/-}$ Rag1$^{-/-}$ as litter mate controls). C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag$^{+/-}$ males were bred with C1galt1$^{f/f}$ IEC Cre$^{-/-}$ Rag1$^{+/-}$ females, and their C1galt1$^{f/f}$ Rag$^{-/-}$ will be selected for future breeding. C1galt1$^{f/-}$ IEC Cre$^{+/-}$ Rag$^{-/-}$ males were bred with C1galt1$^{f/-}$ IEC Cre$^{-/-}$ Rag1$^{-/-}$ females, and their C1galt1$^{f/f}$ Rag$^{-/-}$ will be selected for future breeding. Ultimately, the C1galt1$^{f/f}$ IEC Cre$^{+/-}$ Rag1$^{-/-}$ males were bred with C1galt1$^{f/f}$ IEC Cre$^{-/-}$ Rag1$^{-/-}$ females to maintain the colony as well as for direct experiments.

SUPPLEMENTAL CHAPTER 3

Peroxynitrite Up-Regulates Angiogenic Factors VEGF-A, bFGF, and HIF-1α in Human Corneal Limbal Epithelial Cells

Abstract

Background & Aim

Corneal neovascularization (NV) is a sight-threatening condition associated with excessive ingrowth of blood vessels resulted from deprivation of oxygen in the tissue. Nitric oxide (NO) and superoxide anion (O_2^-) released by macrophages recruited to the corneal react to form the highly toxic molecule peroxynitrite ($ONOO^-$). The overall aim of the manuscript was to investigate the role of $ONOO^-$ in regulating angiogenic factor productionin cultured human corneal limbal epithelial (HCLE) cells. The aim of my contribution to this manuscript was to determine the cytotoxic effects of $ONOO^-$ to HCLE cells.

Methods

To determine the time course of angiogenic factor production by HCLE cells, the cells were incubated with 500 μM of $ONOO^-$ donor for various period of time. The viability of HCLE cells after 500 μM $ONOO^-$ donor treatment was evaluated by 7-AAD staining and flow cytometry.

Results

Treatment with 500 μM of $ONOO^-$ donor for 2, 4, 6, and 8 hours caused minimal HCLE cell death, while treatment for 12 hours caused significant cell death ($p<0.004$).

Conclusion

500 μM of $ONOO^-$ donor treatment did not cause early death of HCLE cells until up to 12 hours, indicating that 500 μM of $ONOO^-$ donor treatment can be used to study HCLE cell angiogenic factor production *in vitro*.

Introduction

Corneal neovascularization (NV) is a sight threatening eye condition that 1.4 million Americans are suffering from annually.[130] Corneal NV is associated with pathological ingrowth of blood vessels from the limbal vascular plexus into the cornea.

Cornea neovascularization depends on the disequilibrium between the pro- and anti-angiogenic factors. [131] Within the cornea, endogenous proangiogenic factors, including vascular endothelial growth factor-A (VEGF) and basic fibroblast growth factor (bFGF), are present at low levels.[132] Following corneal injuries or infections, inflammatory cells infiltrate cornea and lead to an upregulation of proangiogenic factors.[133] Elevated levels of proangiogenic factors may arise indirectly from infiltrating inflammatory cells[134], or from the corneal epithelium.

Besides proinflammatory cytokines, infiltrating macrophages, monocytes, and leukocytes also release reactive oxygen and nitrogen species.[135,136] Nitric oxide (NO) and superoxide anion ($O2^-$) react to form the highly toxic molecule peroxynitrite (ONOO-).[137] Elevated levels of ONOO- are associated with multiple inflammatory diseases[138], and are well-documented in animal models of endotoxin-induced uveitis (EIU) and experimental autoimmune uveitis (EAU),[139,140] but their role in human ocular inflammation is poorly understood.

The angiogenic capacity of $ONOO^-$ has been investigated in smooth muscle cells, vascular endothelial cells, and microvascular endothelial cells.[141] However, the potential role for $ONOO^-$ in corneal neovascularization and its ability to upregulate angiogenic factors, such as VEGF, HIF-1α, and bFGF has not been reported. This study demonstrated that elevated levels of exogenous $ONOO^-$ produce an up-regulation of

these angiogenic factors in a human HCLE cell line. If $ONOO^-$ can stimulate corneal neovascularization *in vivo*, $ONOO^-$ and its donors can be targeted for disease monitoring and treatment.

Materials and Methods

Cell culture

HCLE cells (gift of Dr. Ilene Gibson, Schepens Eye Research Institute, Harvard Medical School) are a spontaneously arising human corneal limbal epithelial cell line. HCLE cells were grown and incubated with keratinocyte serum-free media (KSFM) with human recombinant Epidermal Growth Factor 1-53 and Bovine Pituitary Extract (Life-Technologies, Grand Island, NY) at 37ºC, 5% CO2. HCLE cells were seeded onto 6-well culture plates and incubated for 2 days until cells reached confluence.

$ONOO^-$ treatments

3-morpholinosyndnomine (SIN-1) (≥98% purity, Sigma-Aldrich, St. Louis, MO) has been used as an $ONOO^-$ donor in previous studies.[137,141] The concentrations of $ONOO^-$ used in this study are designed to be within the range used in pathological models of ocular disease[139,142], taking into account that the half-life $ONOO^-$ at pH 7.4 is only a few seconds[137]. Under physiological conditions, endogenous $ONOO^-$ is continuously produced at low concentrations. Thus, higher concentrations and extended exposure times with exogenous $ONOO^-$ are necessary to mimic the biological responses seen with endogenous $ONOO^-$[141]. SIN-1 was added to keratinocyte serum-free medium (KSFM) of cultured HCLE cells at concentrations ranging from 0.125 to 1 mM. SIN-1 was dissolved in KSFM and incubated with HCLE cells after 30 min. It decomposes spontaneously in neutral aqueous media, consuming oxygen to release equimolar amounts of NO and $O2^-$ simultaneously which react rapidly to produce $ONOO^-$.[137] After the incubation with SIN-1 was complete, HCLE cells were washed with

PBS, lysed and boiled at 95 ºC for 5 minutes with Laemmli buffer for western blot, and the conditioned media collected for functional assay studies.

Flow cytometry analysis of cell death

HCLE cells were seeded in 6-well plates at a density of $1x10^6$ cells/well. Once the cells reached 80-90% confluence, they were incubated with 500 µM of SIN-1 for 0, 2, 4, 6, 8, and 12 hours. Samples were stained with 7-amino actinomycin (7-AAD) (BD Biosciences, San Jose, CA) at 0.25ug per million cells in FACS buffer (PBS with 0.5% bovine serum albumin) at room temperature. 7-amino actinomycin D (7-AAD) staining was used to identify and quantitate dead cells by flow cytometry. All samples were immediately acquired on FACSCalibur machine with CellQuest Pro software (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (TreeStar).

Results

Cell Integrity Following 500 μM of SIN-1 Exposure

In order to determine whether 500 μM SIN-1 (an $ONOO^-$ donor) had any cytotoxic effect on HCLE cells, a 7-AAD cell viability assay was performed. When HCLE cells were incubated with 500 μM of SIN-1 for 2, 4, 6, and 8 hours, there was minimal increase in cell death (0.5-2%) compared to the positive control and three different negative controls. The positive controls was generated by incubating the cells in 56ºC for 5 min (44%), and put on ice for another 5 min before staining with 7-AAD. Three negative control conditions were used. First, 2.0 mM of uric acid (UA) was introduced into 1mM SIN-1 + KSFM, 30 min prior to incubation with HCLE cells for 8 hours. Second, KSFM without SIN-1 was used at 0 hour, and HCLE cells were incubated in KSFM alone for 8 hours. Third, 1 mM SIN-1 was added to KSFM media 24 hours prior to incubation with HCLE cells (DCtrl) to create $ONOO^-$ depletion. Significant cell death (**$P<0.004$) was observed when HCLE cells were incubated with $ONOO^-$ for 12 hours (30%). Four independent trials were conducted (Figure S3-1).

Figures and Tables

Figure S3-1

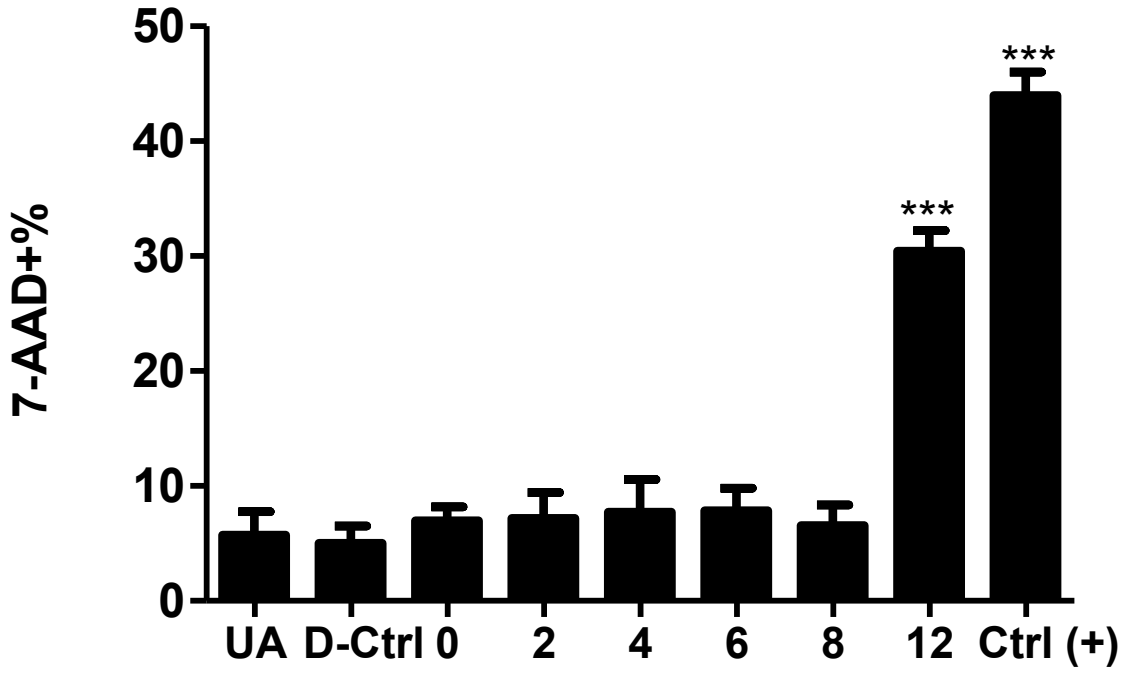

Figure S3-1. ONOO- Does Not Cause Early Cell Death.

HCLE cells treated with 500 μM of SIN-1 for 0-12 hours, and the percentage of dead cells were detected by flow cytometry after 7-AAD staining. Three independent negative controls included: UA (2 mM uric acid + 1mM SIN-1); D-ctrl (depleted SIN-1 donor and KSFM media added in lieu of SIN-1; and 0 hour (no treatment). The positive control (Ctrl (+)) was HCLE cells heat-treated at 56ºC for 5 min, and then incubated on ice for an additional 5min. Cell death was significant (**P<0.005) after 12 hours of treatment with $ONOO^-$.

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