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Natural Killer Cells,

NKG2D, NKR-P1A, and LLT1

by

David Brian Rosen

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

[redacted]

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by
David B. Rosen

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Contributions of Co-Authors to the Presented Work

Chapter 2 of this dissertation is based on material published in *The Journal of Immunology* (2004) 173: 2470-2478, titled, "A structural basis for association of DAP12 with mouse but not human NKG2D" by copyright permission of The American Association of Immunologists. The following co-authors contributed helpful advice or reagents to this work: Jessica A. Hamerman and Taian Chen from UCSF and Manabu Araki & Takashi Yamamura of theDepartment of Immunology, National Institute of Neuroscience, Ogawahigashi, Kodaira, Tokyo). This work was supervised by Lewis L. Lanier.

Chapter 3 is based on material published in *The Journal of Immunology* (2005) 176: 7796-7799, titled, "Cutting Edge: Lectin-Like Transcript-1 Is a Ligand for the Inhibitory Human NKR-P1A Receptor" by copyright permission of The American Association of Immunologists. The following co-authors contributed helpful advice or reagents to this work: Jayaram Bettadapura, Mohammed Alsharifi & Hilary S.Warren from Australian National University and Porunelloor A.Mathew from University of North Texas Health Science Center. This work was supervised by Lewis L. Lanier.

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Natural Killer Cells,

NKG2D, NKR-P1A, and LLT1

by

David Brian Rosen

Lewis L. Lanier, PhD

Abstract

The activity of a Natural Killer (NK) cells is regulated by the collective balance of activating and inhibitory signals from surface receptors. The work described here focuses on the biology of human NK receptors, NKG2D & NKR-P1A.

NKG2D recognizes stress-inducible ligands present on virally infected cells, cancer cells, & cells that have experienced genotoxic damage. While NKG2D does not contain any recognizable signaling motifs, it was previously known to pair with a transmembrane adapter signaling protein, DAP10, to initiate signal transduction events. More recently, studies have revealed that alternative mRNA splicing of the mouse *NKG2D* gene generates receptors that associate with either the DAP10 or DAP12 transmembrane adapter signaling proteins. Here we report that NKG2D function is normal in human patients lacking functional DAP12, indicating that DAP10 is sufficient

for human NKG2D signal transduction. Further, we show that human NKG2D is incapable of associating with DAP12 and provide evidence that structural differences in the transmembrane of mouse and human NKG2D account for the species-specific difference for this immune receptor.

Another C-type lectin whose function has remained largely enigmatic is human NKR-P1A (CD161), present on NK cells and subsets of T cells. In these studies, we demonstrate that lectin-like transcript-1 (LLT1) is a physiological ligand for NKR-P1A. LLT1-containing liposomes bind to NKR-P1A+ cells and binding is inhibited by anti-NKR-P1A mAb. Additionally, LLT1 activates NFAT-GFP reporter cells expressing a CD3ζ-NKR-P1A chimeric receptor; reciprocally, reporter cells with a CD3ζ -LLT1 chimeric receptor are stimulated by NKR-P1A. Moreover, LLT1 on target cells can inhibit NK cytotoxicity and cytokine production via interactions with NKR-P1A.

We further demonstrate that Lectin-like Transcript 1 is expressed on activated lymphocytes. We show that activated primary B cells and many B cell lines express LLT1, activated plasmacytoid dendritic cells and activated monocyte-derived dendritic cells express LLT1, and interestingly, activated T cells seem to express LLT1 as well. Lastly we explore the role of NKR-P1A on T cells and find that while engaging NKR-P1A with antibodies on $CD8^+$ T cells can inhibit TNFα production, NKR-P1A does not seem to play any obvious role in $CD4^+$ T cell cytokine production.

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Chapter One

An Introduction to Natural Killer Cells,

NKG2D, NKR-P1A, and LLT1

Synopsis

The research in this thesis focuses on describing the mechanisms by which Natural Killer cells and other immune cells are regulated by their cell surface receptors. We examined the function of two receptors expressed on NK cells, NKG2D and NKR-P1A. First, we examined NKG2D signal adapter usage to describe how this receptor functions in human and mouse cells. Next we studied the human NKR-P1A receptor, defining Lectin-Like Transcript 1(LLT1) as its physiological ligand, and illustrating how this interaction functions to inhibit natural killer cell activity. Lastly, to further understand the role of the NKR-P1A-LLT1 interaction, we examined the expression pattern of LLT1 in the immune system and probed for the function of NKR-P1A in Natural Killer cells and in the subset of memory T cells that express this receptor.

NK Cells and their Function

Natural Killer (NK) cells were first named for their ability to readily kill cancerous cell lines *in vitro* without prior immunization. In addition to their anti-tumor effects, NK cells play a critical role in anti-viral immunity, particularly against members of the Herpesvirus family. NK cells have also been shown to function in bacterial and parasitic infections (1). NK cells are derived from a common lymphoid progenitor and are thought to originate in the bone marrow; however, new evidence suggests that human NK cells may also develop in lymph nodes (2). NK cells account for 5-10% of the total lymphocyte population and are predominantly found in blood, spleen, and liver, and intriguingly, also in the deciduas of pregnant women, where they might play a role in regulating reproduction (3). NK cells actually represent a quite diverse and

heterogeneous population, with different NK cell subsets expressing different combinations of receptors (4). In humans, three subsets of NK cells have been described: peripheral blood $CD56^{hi}$ $CD16^{-}$ cells, specializing in cytokine production; $CD56^{dim}$ $CD16^{+}$ cells, specializing in cytotoxicity; and uterine $CD56^{hi}$ NK cells, whose function is still largely enigmatic (5).

Unlike T and B cells, which have a single clonotypic antigen receptor dominantly controlling their activity, NK cells have many germline-encoded antigen receptors that regulate their activity, but generation of these receptors does not require genetic rearrangement events. Although NK receptors are either activating or inhibitory, it is fundamentally the balance or integration of total signals from all of the receptor engagement events that will determine the activity of an individual NK cell (6, 7). Whereas engagement of an individual activating receptor alone sometimes results in no effect, ligating multiple activating receptors simultaneously can synergize to give full activation of NK effector functions (8).

Inhibitory NK Receptors

As all normal, healthy nucleated cells in the body express major histocompatibility complex (MHC) class I, which can interact with NK inhibitory receptors, these receptors may exist to control NK reactivity against normal cells, thus limiting autoimmunity in this cell type. However, under conditions of viral infection or oncogenic transformation, many cells lose MHC class I expression, and thus become more susceptible to NK activation and cytotoxicity. If these same cells that now lack MHC Class I express activating ligands for NK receptors, the cells will be susceptible to NK cell-mediated cytotoxicity. This absence of MHC class I leading to NK activation is

deemed the "missing self" hypothesis, which proposes that NK cells can become activated through loss of signaling from inhibitory receptors (9). In reality, a more comprehensive analysis indicates that cells "missing self" are more susceptible to NK cell-mediated killing not only because they lack MHC class I but also because they express ligands that engage activating receptors on NK cells. Red blood cells are an example of a cell type that lacks MHC class I yet does not illicit NK cell-mediated killing, likely due of lack of activating ligands for NK receptors. Although many NK inhibitory receptors recognize MHC class I, certain NK inhibitory receptors recognize non-MHC ligands (10, 11).

Inhibitory NK receptors include "long" Killer cell Immunoglobulin-like Receptor (KIR) proteins, e.g. KIR2DL and KIR3DL molecules, named for their long cytoplasmic tails containing one or more ITIM (Immunoreceptor Tyrosine-Based Inhibition Motif) that mediates their inhibitory function by recruiting phosphatases such as SHP-1, SHP-2, and SHIP (6, 12, 13). Human inhibitory KIR recognize MHC class I and are thought to be analogous to the inhibitory Ly49 receptor family in mice. Other examples of inhibitory receptors expressed by human NK cells include: LAIR-1, which recognizes collagens (14); MAFA (also designated KLRG1), which recognizes e-cadherins (15, 16); NKG2A, which in complex with CD94 recognizes HLA-class I leaders presented by HLA-E (17); and ILT-2, which recognizes conserved epitopes in the α3 domain of several MHC class I proteins (18, 19).

Table 1.1 Examples of Human NK Activating Receptors

Receptor	Cellular Ligand	Transmembrane Signaling Adapter	Pathway
KIR2DS	HLA-C	DAP12	ITAM
KIR3DS	???	DAP12	ITAM
NKG2D	MICA/B, UBLP1-4	DAP10	PI3K
NKG2C/CD94	HLA-E with peptide	DAP12	ITAM
NKp30	???	FcεRIγ or CD3ζ	ITAM
NKp46	???	FcεRIγ or CD3ζ	ITAM
NKp44	???	DAP12	ITAM
CD16	IgG	FcεRIγ or CD3ζ	ITAM
2B4	CD48	None	SAP
DNAM-1	CD112, CD155	None	PKC
CD96	CD155	None	???

Activating NK receptors

NK activating receptors initiate effector functions by various mechanisms (Table 1.1). Certain activating receptors, such as NKG2D and the "short" KIR, e.g. KIR2DS and KIR3DS molecules, lack intrinsic signaling motifs and interact with signaling adapter proteins through transmembrane charges to relay activating signals into the cells. Transmembrane-anchored signaling adapter proteins expressed by NK cells include:

DAP10, DAP12, CD3ζ, and FcεRIγ. Other NK activating receptors that use transmembrane anchored signaling adapter proteins include the NKG2C/CD94 complex, and the "natural cytotoxicity" receptors (NKp30, NKp44, and NKp46), and CD16, the Fc receptor for IgG responsible for antibody-dependent cellular cytotoxicity (ADCC). NK activating receptors also include 2B4 (CD244), which recognizes CD48 and signals via the intracellular SAP adapter protein, DNAM-1 (CD226), which recognizes CD112 and CD155, and CD96, which recognizes CD155 (7). Although many of these receptors lack identified ligands, some such as NKG2D have been shown to recognize well-characterized cell surface proteins (ligands) induced by cellular stress, such as transformation, DNA damage, or viral infection. In the mouse, there are examples of NK receptors, such as Ly49H, which directly interact with viral proteins, giving NK cells the ability to recognize and attack virally infected cells (20).

NK cell effector functions

NK cells serve many functions. As suggested by their name, Natural Killer cells have killing or cytotoxic potential. NK cell-mediated cytotoxicity is achieved through directed release toward target cells of secretory granules containing cytotoxic proteins such as perforin and granzymes. Perforin has the ability to form pores or holes in cell membranes, disrupting cellular integrity and allowing granzymes to enter the target cell and initiate a proteolytic cascade that will activate caspases and the apoptotic program (21-23). The exact mechanism by which perforin enables granzyme entry into target cells is unclear, but may require an active role by target cell membrane repair machinery (24) The degranulation of NK cells seems to be particularly dependent on PLC-γ and calcium signaling. NK cells can also perform cytotoxicity through the expression of TNF

family members such as FAS Ligand (FAS-L), TRAIL, or soluble TNFα, which can engage TNF receptors on target cells and initiate the apoptotic cascade (25).

NK cells also produce cytokines and chemokines that serve to recruit and activate other immune cells. NK cells produce interferon-γ (IFN γ), which serves to activate macrophages and T lymphocytes and direct the immune system toward a cell-mediated response. NK cells also make MIP-1 β, which can recruit macrophages, and TNFα, which promotes inflammation and contributes to cytotoxicity. The activity of NK cells can be enhanced by soluble cytokines, such as type-I IFN, IL-12, and IL-15 produced by other immune cells. Thus, the primary effector functions of NK cells, i.e. cytokine secretion and cytotoxicity, serves to control viral infections and outgrowth of transformed cells through recognition and elimination of infected, transformed, or stressed/damaged cells.

NKG2D

NKG2D is a C-type lectin activating receptor expressed on virtually all NK cells, as well as all human $CD8^+$ T cells, activated mouse $CD8^+$ T cells, and invariant NKT cells (26, 27). Unlike the other NKG2 family members (i.e. NKG2A and NKG2C), NKG2D does not associate with CD94. NKG2D is fairly distant in structure and function from NKG2A, C, and E. Like other C-type lectins, NKG2D is expressed on the cell surface as a type II disulfide-bonded dimer. NKG2D requires association with signal adapter proteins, such as DAP10, for stable surface expression. This will be discussed in detail in Chapter 2. NKG2D recognizes MHC-like molecules that are induced by cellular stress, DNA damage, viral infection, or oncogenic transformation, thereby effectively

marking unhealthy cells for elimination by NK cells through the NKG2D pathway. NKG2D ligands do not associate with beta-2-microglobulin and do not present peptides. In humans, NKG2D ligands include the highly polymorphic MICA and MICB proteins (26), as well as ULBP (UL-16 Binding Protein)1, ULBP2, ULBP3,ULBP4, and RAET1G (28-30).

Interestingly, the ULBPs were found and named for their ability to bind UL16 (UL16 Binding Protein), a human cytomegalovirus-encoded protein that serves to degrade NKG2D ligands and thus evade NKG2D-mediated immunity (28). Evading NK cells by downmodulating or masking NK receptor ligands, such as NKG2D ligands, turns out to be a common theme with viruses, especially Herpesviruses such as mouse and human cytomegalovirus (31).

On NK cells, NKG2D can potently induce cytotoxicity and can synergize with other receptors to induce cytokine secretion. There have been roles proposed for NKG2D on T cells; however, not all groups have been able to demonstrate this effect (32, 33). In addition to controlling viral infections and tumorigenesis, NKG2D may contribute to autoimmune diseases such as diabetes (34), rheumatoid arthritis (35, 36), and celiac disease (37-39). Chapter 2 examines the mechanisms responsible for mouse and human NKG2D-mediated signal transduction.

NKR-P1A and LLT1

NKR-P1A, also known as CD161 or KLRB1, is a C-type lectin receptor present on 70% of human peripheral blood NK cells, invariant NKT cells, γδ T cells, and subsets of effector/memory αβ -TcR T cells, including both $CD4^+$ and $CD8^+$ T cells (40) NKR-

P1A, like NKG2D, is a type II disulfide-bonded homodimer. Rodents have several *Klrb1* (also named *Nkrp1*) genes encoding either activating or inhibitory NK receptors of the C-type lectin-like superfamily, including NKR-P1C -the prototypic NK1.1 antigen defining mouse NK cells in C57BL/6 mice (41). By contrast, only a single, non-polymorphic gene in the Nkrp1 family, designated *KLRB1*, exists in the human genome (40). Human NKR-P1A is more abundantly expressed on T cells than is mouse NK1.1.

Functionally, NKR-P1A has been implicated in the transendothelial migration of $CD4^+$ T cells (42, 43). In addition, anti-NKR-P1A monoclonal antibodies (mAb) have been reported to costimulate the anti-CD3 mAb-induced proliferation of human CD1d-specific NKT cells (44), and to induce proliferation of immature thymocytes (45). Additionally, cross-linking with an anti-NKR-P1A mAb potently inhibits human NK cell-mediated cytotoxicity against FcR^+ target cells (46, 47). The function of NKR-P1A on $CD4^+$ and $CD8^+$ memory T cells has not been elucidated to date, although prior studies with some NKR-$P1A^+$ T cells suggest a stimulatory role.

Recently, ligands for two mouse NKR-P1 family members have been identified (48, 49). The activating mouse NKR-P1F receptor recognizes Clr-g (encoded by the *Clec2i* gene) (48), and the inhibitory mouse NKR-P1D recognizes Clr-b (encoded by the *Clec2d* gene) (49). Another name for Clr-b is osteoclast inhibitory lectin (OCIL), as it was independently identified as an osteoblast-derived glycoprotein, which in soluble form inhibits *in vitro* osteoclastogenesis (50, 51)

Although mice have multiple *Clec2* genes, only one ortholog, designated *CLEC2D* (also named lectin-like transcript-1, LLT1), exists in humans. Like mouse Clr-b, human LLT1 was independently identified as an osteoblast-derived protein (designated

osteoclast inhibitory lectin, OCIL), which may block osteoclast differentiation (50, 51). In addition to reports describing LLT1 expression in osteoblast lines, some data exist demonstrating that LLT1 may be expressed by PMA-activated peripheral blood lymphocytes (52). Human *CLEC2D* and the mouse *Clec2* genes are closely genetically linked to the human *KLRB1* gene and the mouse *Klrb* loci, respectively, in the "NK complex", a genomic region containing many C-type lectin NK receptors on human chromosome 12 and mouse chromosome 6. Moreover, the proteins encoded by the mouse and human Clec2 gene families are type II proteins of the C-type lectin-like superfamily. As such, LLT1 represented an attractive candidate ligand for human NKR-P1A.

The work described here explores how NK receptors function to regulate cellular responses. Chapter 2 focuses on signaling mechanisms by activating receptor, NKG2D. Chapter 3 defines the ligand specificity of NKR-P1A and illustrates how LLT1 interacts with NKR-P1A to inhibit NK cytotoxicity. Chapter 4 explores the expression pattern of LLT1 and examines the function of NKR-P1A on T cells. Chapter 5 describes the significance of these results and suggests further directions for experimentation.

In addition to the experiments described here, I have performed research in collaboration with other scientists examining the biology of leukocyte cell surface receptors from both at UCSF and University of Texas MD Anderson Cancer Center, which resulted in the following publications:

Voehringer D, **Rosen DB**, Lanier LL, Locksley RM. CD200 receptor family members represent novel DAP12-associated activating receptors on basophils and mast cells. 2004. *Journal of Biological Chemistry*. 279 (52): 54117.

Shiow LR, **Rosen DB**, Brdickova N, Xu Y, An J, Lanier LL, Cyster JG, Matloubian M. CD69 acts downstream of IFN□□□ to inhibit $S1P_1$ and lymphocyte egress from lymphoid organs. 2006. *Nature*. 440 (7083): 540.

Cao W, **Rosen DB,** Ito T, Bover L, Bao M, Watanabe G, Zhang L, Lanier LL, Liu YJ. Plasmacytoid Dendritic Cell-Specific Receptor ILT7/Fc□RI□ Inhibits Toll-Like Receptor-Induced Interferon Production. 2006. *Journal of Experimental Medicine*. 203 (6): 1399.

Carr WH, **Rosen DB,** Arase H, Nixon DF, Michaelsson J, Lanier LL. Cutting Edge: *KIR3DS1*, an AIDS-restrictive gene, encodes a receptor that is expressed on peripheral blood NK cells and triggers NK cell activation. 2007. *Journal of Immunology*. 178 (2): 647

Cao W, Zhang L, **Rosen DB**, Bover L, Watanabe G, Bao M, Lanier LL, Liu YJ. Plasmacytoid Dendritic Cell Receptor BDCA2/Fc□RI□ Complex Uses BCR-like Signaling Cascade to Modulate Toll-Like Receptor Responses. 2007. *Public Library of Science: Biology*. 5(10): e248

Chapter Two

Differential Signaling Adapter usage by

Mouse and Human NKG2D

Abstract

To understand how NK cells are regulated, our laboratory has been studying the biology of specific NK cell activating receptors, such as NKG2D. To understand the function of NKG2D, we are interested in knowing how this receptor sends activating signals into the cell. Recently, studies have revealed that alternative mRNA splicing of the mouse *NKG2D* gene generates receptors that associate with either the DAP10 or DAP12 transmembrane adapter signaling proteins. Here we report that NKG2D function is normal in human patients lacking functional DAP12, indicating that DAP10 is sufficient for human NKG2D signal transduction. Further, we show that human NKG2D is incapable of associating with DAP12 and provide evidence that structural differences in the transmembrane of mouse and human NKG2D account for the species-specific difference for this immune receptor. The experiments detailed in this chapter were published in the *Journal of Immunology* (2004) 173: 2470-2478.

Introduction

The activity of NK cells is regulated by a balance of positive and negative signals transduced, respectively, via activating and inhibitory cell-surface receptors (20, 53). The activating receptor NKG2D, a C-type-like lectin and type II transmembrane protein (54), is expressed on all NK cells, γδ-TCR$^+$ T cells, and human CD8$^+$ T cells and is induced on activated mouse CD8$^+$ T cells (55). NKG2D recognizes several major histocompatibility complex (MHC)-related ligands, including the ULBP and MIC family of proteins in humans (26, 28) and H60, RAE-1, and MULT1 in mice (56-59). These NKG2D ligands, although usually absent from or expressed at low levels by normal adult tissues, are often induced on stressed, infected, or tumor cells in adult life (reviewed in (60)). In this fashion, leukocytes expressing NKG2D can directly recognize transformed or infected cells.

Activation through NKG2D can have multiple outcomes including the production of IFN-γ and the triggering of cell-mediated cytotoxicity (26, 61). In αβ-TCR$^+$ T cells, NKG2D has been suggested to provide a costimulatory role similar to CD28, enhancing TCR-mediated signaling events (32, 62). *In vivo* NKG2D is involved in anti-tumor as well as anti-viral immunity (reviewed in (63)).

NKG2D itself lacks intrinsic signaling capabilities. Like the T cell antigen receptor (TCR), NKG2D contains a positively charged amino acid within its transmembrane region and requires association with adapter signaling proteins for cell-surface expression. These adapter proteins contain complementary negatively charged amino acids within their transmembrane regions that form a salt bridge with NKG2D. Both in mice and humans, NKG2D homodimers associate with and signal through

homodimers of the transmembrane adapter protein DAP10 (61, 64). Signaling through DAP10 involves phosphorylation of its cytoplasmic YxxM motif, recruitment of the p85 subunit of phosphatidylinositol-3 kinase (PI3K), and downstream signaling through AKT (64-66).

In mice, alternative mRNA splicing generates two functionally distinct isoforms of the NKG2D protein (61). The mouse NKG2D-Long (mNKG2D-L) protein comprises 232 amino acids, whereas the NKG2D-Short (mNKG2D-S) protein lacks the first 13 N-terminal amino acids and initiates translation at a second methionine in the cytoplasmic domain of this type II protein. This shorter isoform is expressed in activated, but not resting, mouse NK cells and is capable of pairing with homodimers of either DAP10 or DAP12, an ITAM-containing transmembrane adapter protein. Like other ITAM sequences, DAP12's intracellular $YxxL_{6-8x}$ YxxL/I motif recruits Syk family kinases (67). Mouse NKG2D-L (mNKG2D-L) pairs exclusively with DAP10 (61). Both DAP10 and DAP12 signaling contribute to NK cell-mediated cytotoxicity, whereas DAP12 signaling also stimulates cytokine production, such as IFN-γ (61, 64, 68, 69).

The ability of the mouse NKG2D to generate identical receptors with distinct signaling properties by virtue of association with different adapter proteins prompted the question of whether human NKG2D also demonstrates this property. Here, we have examined NKG2D expression and functional activity in human PBMCs of patients lacking a functional *DAP12* gene and have explored the structural basis for association of human and mouse NKG2D with the DAP10 and DAP12 adapter proteins.

Materials and Methods

Characterization of Nasu-Hakola Patients

The *DAP12* (*tyrobp)* gene in Nasu-Hakola patient designated NH1 has a single base mutation in the start codon of exon 1 that has recently been identified in Japanese patients (70). Patients NH2 and NH3 have a single base deletion in exon 3 (70, 71). Loss of DAP12 protein expression in these patients was confirmed by Western blotting as previously described (70). Studies of all these subjects were conducted according to the institutional guideline.

Cytotoxicity assays

PBMC were prepared from peripheral blood samples of healthy individuals and three patients with Nasu-Hakola disease by density gradient centrifugation by using Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMC were resuspended at 2 x 10^6/ml in RPMI-1640 medium supplemented with 10% of FCS, 2 mM L-glutamine, Hepes, penicillin, streptomycin, and 2-ME. PBMC were placed into 24-well plates in the presence of 100 U/mL (for mAb-induced redirected cytolytic assays) or 1000 U/ml (for BaF/3 cytotoxicity assays) recombinant IL-2 (rIL-2) (Shionogi, Osaka, Japan). A lower concentration of IL-2 was used for assays with P815 target cells because high doses of IL-2 causes considerable background cytotoxicity against P815, likely due to LAK activity. Activated PBMC were harvested after 48 h-culture, and then used in 4-h ^{51}Cr release cytotoxicity assays as described (72). BaF/3, BaF/3 cells expressing MICA*0019, and FcR^+ P815 cells were labeled with 100 μCi ^{51}Cr (Perkin Elmer, Boston, MA, USA) for 2 h at 37ºC, washed three times, and used as target cells in cell-mediated

cytotoxicity assays. For mAb-induced re-directed cytotoxicity assays using P815 target cells, PBMC were cultured in the presence of media only, control mAb anti-CD56 mAb (Leu 19, Becton Dickinson Immunocytometry Systems, San Jose, CA), or anti-NKG2D mAb (made in collaboration with Dr. J.P. Houchins, R&D Systems, Minneapolis, MN). mAbs were used at a final concentration of 2.5 μg/ml.

cDNAs, chimeras and plasmids

Human "short" NKG2D, human truncated NKG2D, mouse truncated NKG2D, mouse-human NKG2D chimeras (mTM hEC), and NKG2D-CD69 chimeras were created by standard PCR mutagenesis. TM regions were determined by using the following TM prediction programs and a consensus sequence was obtained by comparison of the analyses: Tmap (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+Launch+-id+1dvsK1MjgHZ+-appl+tmap+-launchFrom+top), DAS (http://www.sbc.su.se/~miklos/DAS/), Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html), HMMTOP (http://www.enzim.hu/hmmtop/html/submit.html), SOSUI (http://sosui.proteome.bio.tuat.ac.jp/cgi-bin/sosui.cgi?/sosui_submit.html), & TMHMMM (http://www.cbs.dtu.dk/services/TMHMM/). Protein chimeras and truncations were created utilizing the following amino acids: mΔNKG2D begins at K48, hΔNKG2D at R47 and ΔCD69 at V39 (with numbering beginning at the first amino acid in the mouse NKG2D-L isoform and the human NKG2D and CD69 sequences). Mouse NKG2D TM construct consists of amino acids K48-V84 and the human NKG2D TM construct spans R47-N80. Human NKG2D extracellular domain (EC) begins at L82, and the human CD69 EC at G65. The human NKG2D "short" intracytoplasmic (IC) region construct consists of M15-E48, the human Long IC construct of M1-E48, and the mouse NKG2D tail construct spans M1-E13. CD69 cDNA was synthesized from mRNA isolated from Jurkat T cells stimulated 24 h with 25 ng/mL PMA (Calbiochem Darmstadt, Germany) by reverse transcription with Superscript II (InVitroGen Carlsbad, CA). Site-directed mutagenesis was performed by using a Quick Change kit (InVitroGen), according to the

manufacturer's instructions. All constructs were confirmed by DNA sequencing. cDNAs were subcloned into pMX-pie (containing a puromycin resistance gene, an internal ribosomal entry site (IRES) element, and the enhanced green fluorescent protein (GFP) gene) or pMX-puro retroviral vectors (73).

Cells and transfectants

Plasmid constructs were transfected with Lipofectamine 2000 (InVitroGen) into the Phoenix packaging cell lines (generous gifts from Dr G. Nolan, Stanford University, Palo Alto, CA) (74) to produce retroviruses. Retroviruses in medium containing 8 μg/mL polybrene (Sigma) were used to infect BaF/3 reporter cells. Briefly, polybrene was added to retroviral supernatants, and the mixture was used to resuspend BaF/3 reporter cells. Following centrifugation of the cells in the retrovirus-containing medium (1300 x g for 2.5 h), transfected reporter cells were incubated for 48 h at 37ºC and then assayed for transgene expression. BaF/3 reporter cells were created from the mouse pro-B BaF/3 cell line. Dr. S. Tangye (Centenary Institute, Sydney, Australia) generously provided BaF/3 cells transfected with a mouse IL-3 cDNA to permit autocrine production of this requisite growth factor. To create Myc-DAP10 reporter cells, IL-3$^+$ BaF/3 cells were infected with retroviruses (pMX-puro vector) containing a cDNA including the human CD8 leader segment, followed by the Myc epitope (EQKLISEEDL) joined to the extracellular N-terminal domain of human DAP10 (75). Similarly, to create Flag-DAP12 reporter cells, IL-3$^+$ BaF/3 cells were infected with retroviruses (pMX-puro vector) containing cDNA including the human CD8 leader segment, followed by the Flag epitope (DYKDDDDK) joined to the extracellular N-terminal domain of human DAP12, as described (67, 76). Infected cells were then selected in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 1 μg/ml puromycin.

Flow cytometry and antibodies

For immunofluorescence analysis of transfected myc-DAP10 BaF/3 reporter cells, 1 x 10^6 cells were stained with anti-myc mAb 9E11 (generously provided by Dr. G. Evan, UCSF), followed by a donkey anti-mouse IgG secondary antibody conjugated to phycoerythrin (PE) (Jackson Immunoresearch Laboratories, West Grove, PA). Cells were then pre-incubated in 10% normal mouse serum (Sigma) before being stained with either biotin-conjugated mouse anti-human NKG2D mAb (clone 149810, R&D Systems), biotin-conjugated rat anti-mouse NKG2D mAb CX5, or allophycocyanine (APC)-conjugated mouse anti-human CD69 mAb Leu 23 (PharMingen). Biotin-conjugated antibodies were detected with CyChrome-conjugated or APC-conjugated streptavidin (PharMingen). For immunofluorescence analysis of transfected Flag-DAP12 BaF/3 reporter cells, 1 x 10^6 cells were stained with biotin-conjugated anti-Flag mAb M2 (Sigma), followed by CyChrome-conjugated streptavidin (PharMingen). Cells were also stained with PE-conjugated anti-mouse NKG2D (CX5), anti-human NKG2D PE, or anti-human CD69 (Leu 23) (PharMingen), followed by donkey anti-mouse IgG PE. Live cells were gated based on forward and side scatter profiles. Retrovirus-infected cells were gated based on GFP fluorescence. Cells were analyzed by using a FACSCalibur (Becton Dickinson, San Jose, CA) or a small desktop Guava® Personal Cytometer with Guava ViaCount™ and Guava Express™ software (Hayward, CA).

Immunoprecipitations and Western Blots

50 x10^6 transfected BaF/3 cells were solubilized in 1mL Brij-NP-40 lysis buffer (0.875% Brij 97, 0.125% NP-40, 10 mM Tris base, 150 mM NaCl, and protease inhibitors, all from Sigma). Cell lysates were pre-cleared with 60 μL Protein G Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. Anti-human NKG2D (clone 149810,

R&D Systems) or an isotype-matched control mAb were crosslinked to Protein G beads by incubation in PBS for 30 min, followed by incubation in 10 mM dimethyl pimelimidate dihydrochloride (DMP, Pierce Chemicals), 200 mM triethanolamine (Sigma) pH 8.2 for 45 min and extensive washing. mAb-coated beads were used for immunoprecipitation of pre-cleared lysates for 3 h at 4°C. After washing, immunoprecipitates were eluted by adding non-reducing sample buffer and incubating for 30 min at room temperature. 2-ME (Sigma) was added, samples were boiled, and analyzed by 15% SDS-PAGE. Samples were transferred to Immobilon P membrane (Millipore), blocked, and probed with goat anti-human DAP10 antibody N-17 (Santa Cruz Biotech) or anti-human DAP12 mAb DX37 (75), followed by horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (Amersham Pharmacia Biotech) or goat anti-mouse IgG (Amersham Pharmacia Biotech), respectively, and visualized with chemiluminescent substrate (Pierce Chemical Co.)

Results

PBMCs from DAP12$^{-/-}$ patients display normal NKG2D function

Only the counterpart of the mouse NKG2D-Long isoform has been described in humans and has been shown to co-immunoprecipitate with DAP10, but not DAP12 (64, 75). Nonetheless, this does not exclude a weak or indirect association between human NKG2D and DAP12 that may contribute to NKG2D receptor function in human lymphocytes. Therefore, studies were undertaken to address formally a potential role for DAP12 in human NKG2D receptor-dependent NK cell activation. Nasu-Hakola disease, also called polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), is a globally distributed recessively inherited disease caused by loss-of-function mutations in the *DAP12* (also named *TYROBP*) gene (71). Here, we analyzed peripheral blood mononuclear cells (PBMC) from three Japanese Nasu-Hakola patients (NH patients) for their NKG2D-mediated cytolytic function. Each of these patients has a single point mutation in his/her *DAP12* genes that causes premature stop codons and results in a complete lack of detectable DAP12 protein (see Materials and Methods). We examined the peripheral blood CD3-, CD56+ NK cells of these patients and found that the expression of NKG2D on the cell surface of their NK cells was indistinguishable from normal, healthy individuals (Fig. 2.1A). Previously, we reported that loss-of-function mutations in human *DAP12* did not affect expression of the closely linked *DAP10 (HCST)* gene (71); however, in this prior study NKG2D receptor-dependent functions were not directly analyzed.
In order to formally address the activity of the NKG2D receptor in patients lacking DAP12, we tested the ability of PBMCs from these patients to kill target cells by the NKG2D-dependent pathway. This was achieved by using an anti-NKG2D mAb-induced re-directed cytotoxicity assay and by using mouse target cells transfected with a human NKG2D ligand, MICA*0019. PBMCs from the three NH patients, as well as a normal, healthy individual, were cultured for 48 h in IL-2 and then used as effector cells in these cytotoxicity

assays. Using ^{51}Cr-labeled FcR^{+} P815 target cells, anti-NKG2D mAb induced cytotoxicity mediated by PBMCs of healthy individuals and NH patients, whereas the anti-CD56 mAb used as a negative control failed to augment lytic activity (Fig. 2.1B). No significant difference was seen in NKG2D-mediated cytotoxicity levels between the patient and control cells. To further characterize NKG2D function in NH patients, we performed cytotoxicity assays against BaF/3 mouse pro-B cells stably transfected with MICA*0019, a physiological ligand of human NKG2D. While minimal cytotoxicity was seen against untransfected, parental BaF/3 cells, BaF/3 cells stably expressing MICA stimulated elevated cytotoxicity from activated PBMCs of both normal individuals and NH patients. No significant difference was observed in cytotoxicity levels mediated by PBMCs of healthy individuals and NH patients against MICA-bearing target cells. Thus, NKG2D expression on NK cells and NKG2D-dependent functions were indistinguishable in healthy individuals and the NH patients. These experiments demonstrate normal human NKG2D function in the absence of DAP12.

Fig 2.1

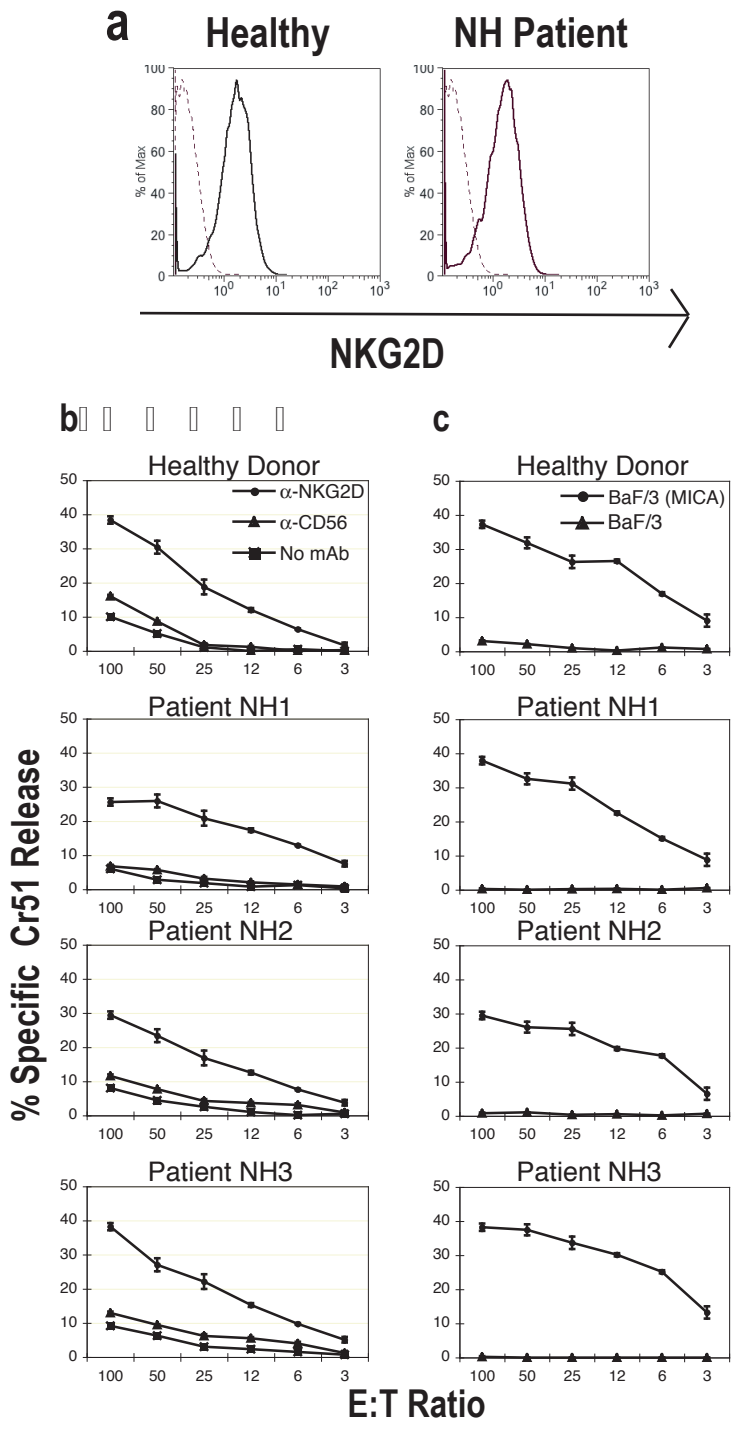

FIGURE 2.1. NKG2D expression and function in Nasu-Hakola patients.

(A) NKG2D levels on NK cells are identical in normal healthy humans and NH patients. CD3-, CD56+ NK cells from PBMC of a normal, healthy individual and Nasu-Hakola patients (NH1, NH2, and NH3) were stained with mAb against CD3, CD56 and NKG2D or appropriate control Ig and were analyzed by flow cytometry. Representative data from one NH patient are shown (dashed lines, isotype-matched Ig control; bold lines, anti-NKG2D).

(B) PBMC from a normal, healthy individual and three Nasu-Hakola patients (NH1, NH2, and NH3) were cultured for 48 h in IL-2 and assayed for mAb-induced re-directed cytotoxicity against FcR+ P815 target cells in the absence or presence of anti-NKG2D mAb or anti-CD56 mAb (used as a negative control).

(C) IL-2 activated PBMC from a normal, healthy individual and three Nasu-Hakola patients (NH1, NH2, and NH3) were assayed for cytolytic activity against mouse Ba/F3 target cells or Ba/F3 cells stably transfected

Unlike mouse NKG2D-S, human NKG2D does not associate with DAP12

Many NK receptors, including NKG2D, KIR2DS, Ly49D, NKR-P1C, NKp30, NKp44, NKp46, and CD94/NKG2C, are multi-subunit receptor complexes that convey signals via the transmembrane adapters FcεRIγ, CD3ζ, DAP10, or DAP12 (reviewed in (6)). All of these adapters have a negatively charged aspartic acid residue in their hydrophobic transmembrane domain, which is critical for interaction with an oppositely charged basic residue in their associated ligand-binding receptors. For NKG2D, this basic residue is a conserved arginine in the transmembrane region (Fig. 2.2A, starred residue). The human and mouse NKG2D-L receptors have been shown to pair and signal through DAP10, but not DAP12 (61, 64).

Recently, a second isoform of mouse NKG2D was discovered that lacks the first 13 N-terminal cytoplasmic amino acids and uses an alternative methionine start site due to alternative splicing of the transcript (61, 77). This shorter mouse NKG2D isoform is capable of pairing and signaling with both DAP10 and DAP12 adapters (61, 77). Examination of the predicted amino acid sequence of human NKG2D reveals that it also contains a second N-terminal methionine residue that could potentially act as an alternative start site (Fig. 2.2A). Although to date no alternatively spliced human transcript involving the NKG2D cytoplasmic domain has been identified (78), it is impossible to exclude the existence of such isoforms at a low abundance or that they are only expressed in certain conditions of activation or in selected cell types. Therefore, we have addressed the issue using a different approach. Here, we ask the question of whether a theoretical short human NKG2D isoform lacking the first 14 amino acids, is able to pair with DAP12.

Fig 2.2

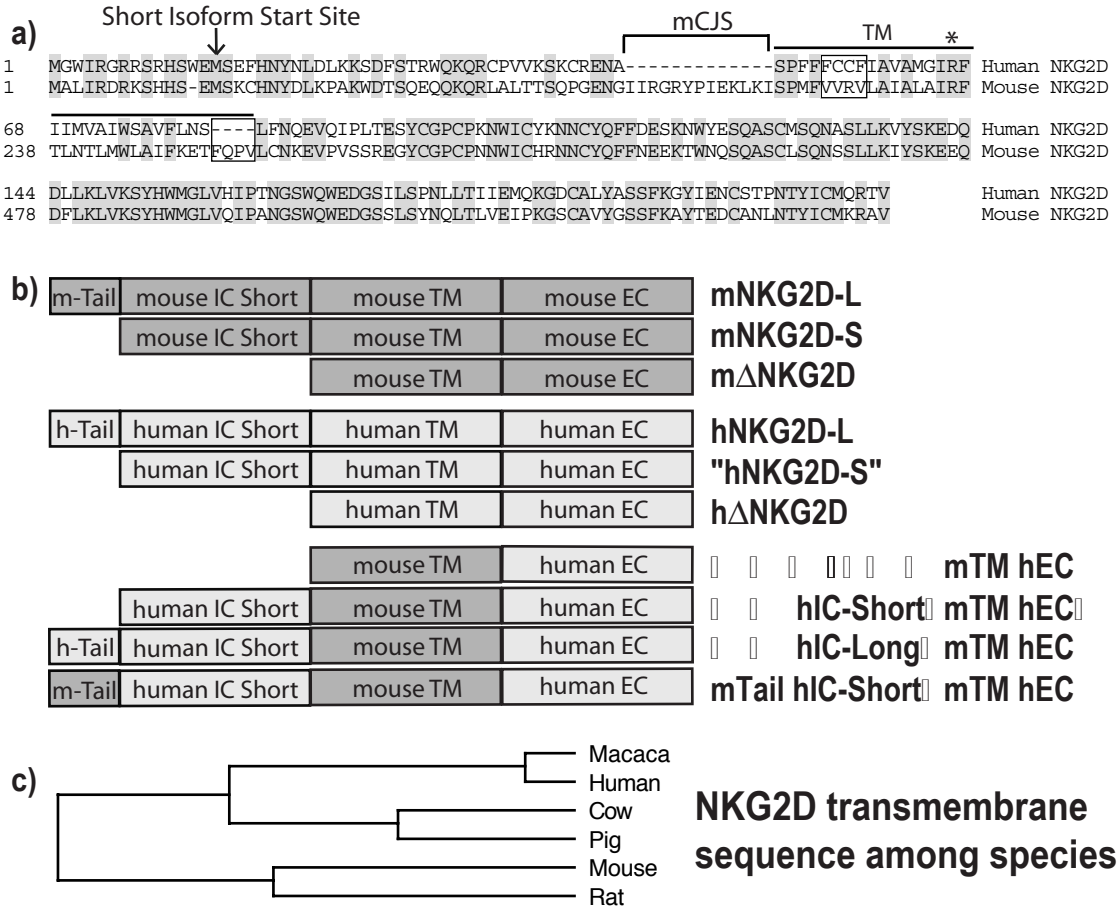

FIGURE 2.2. Human and mouse NKG2D comparisons and receptor constructs.

(A) Alignment of predicted mouse and human NKG2D amino acid sequences. Identical residues in the mouse and human proteins are shaded. An arrow indicates the methionine (M) beginning of the mouse NKG2D-short isoform and the artificial human "NKG2D-short" protein. The murine cytoplasmic juxtamembrane sequence (mCJS), present in mouse, but not human NKG2D, is in brackets. The putative transmembrane region is over lined and a star indicates the conserved arginine (R) residue required for association with DAP10(Wu, Song et al. 1999). Putative transmembrane regions were defined as the consensus from six transmembrane prediction programs (See Materials and Methods section). Boxed are regions and residues of interest.

(B) Schematic representation of the truncations and chimeric proteins generated, with the boundaries as defined in (the Materials and Methods).

(C) Phylogenetic comparison of the transmembrane regions of NKG2D in the indicated species, as determined by Clustal W analysis of the proteins using MegAlign (DNASTAR Software).

To address this experimentally, we artificially created a short human NKG2D using the second methionine residue as the start site and tested its ability to pair with DAP10 and DAP12. For these assays, we used mouse BaF/3 reporter cells stably transfected with Myc epitope-tagged human DAP10 or with Flag epitope-tagged human DAP12. In the absence of an associated receptor, Myc-DAP10 and FLAG-DAP12 were expressed at only low levels on the cell surface of the reporter cells. These reporter cells were infected with retroviruses encoding the wild-type human NKG2D (designated hNKG2D-L), the artificially created human NKG2D-Short ("hNKG2D-S"), the mouse NKG2D-Long (mNKG2D-L), the mouse NKG2D-Short (mNKG2D-S), or an empty retroviral vector. The pMX-pie retroviral vector used in these experiments harbors an IRES-GFP element downstream of the inserted cDNA, allowing for infected cells to be readily detected by the expression of green fluorescence.

Cells were stained with mAbs against the appropriate epitope tags and either human or mouse NKG2D. Infected cells, detected by gating on GFP-positive cells using flow cytometry, were then analyzed for co-expression of NKG2D and the epitope-tagged adapter protein of interest. Coordinate expression of NKG2D and its associated adapter protein on the surface of transfected cells is indicated by the "diagonal" relationship observed in the bivariate dot plots. The "long" NKG2D proteins of both species and the mNKG2D-S protein paired with DAP10, as would be expected based on prior reports (61, 64) (Fig. 2.3). In accordance with prior findings (61, 77), mNKG2D-S efficiently paired with DAP12. By contrast, whereas the "hNKG2D-S" associated with DAP10, it was completely unable to pair with DAP12. Human and mouse NKG2D-L were able to pair interchangeably with either human or mouse DAP10, without species preference

(data not shown). Similarly, mNKG2D-S associated equally with mouse or human DAP12, whereas "hNKG2D-S" failed to assemble with either mouse or human DAP12. Collectively, these results demonstrate a fundamental difference between the human and mouse NKG2D proteins, rather than species-specific differences in the conserved adapter proteins.

Fig 2.3

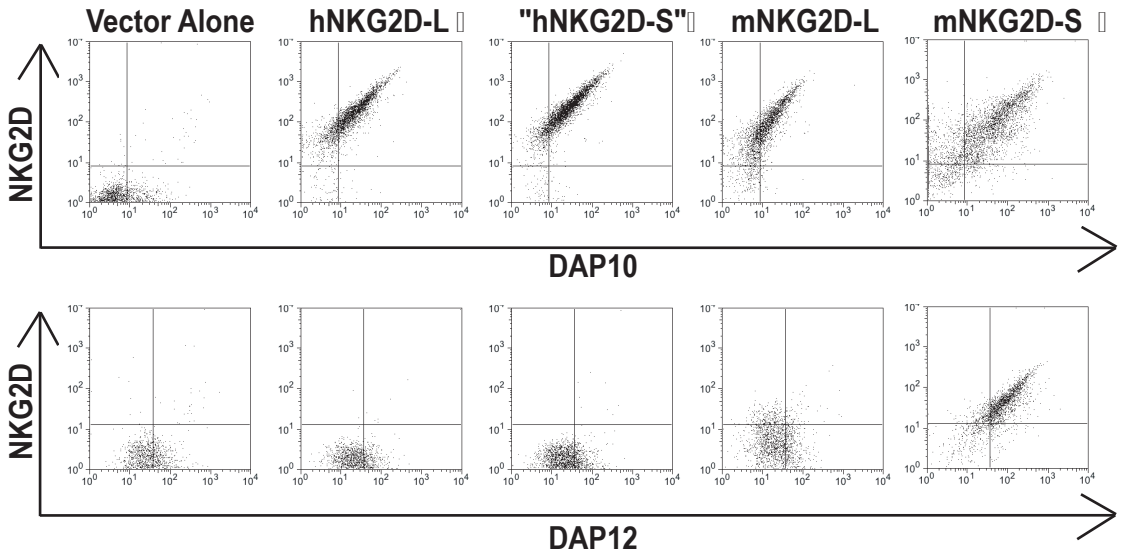

FIGURE 2.3. Unlike mouse NKG2D-S, human "NKG2D-S" does not pair with DAP12.

Ba/F3 reporter cells stably expressing Myc-DAP10 (upper panels) or Flag-DAP12 (lower panels) were infected with retroviruses with the indicated NKG2D constructs in a vector containing an IRES-GFP element. Samples were stained with the relevant anti-NKG2D and anti-epitope tag mAbs and analyzed by flow cytometry. Data shown are gated on GFP+ cells, which confirmed expression of the construct in the infected cells. Results shown are representative of at least three independent experiments.

Mouse NKG2D cytoplasmic domain is not required for DAP12 association

In mouse NKG2D, the 13 amino acid N-terminal cytoplasmic tail present in NKG2D-L, absent in mNKG2D-S, abrogates DAP12 binding (61). Similar to mouse NKG2D, the first 14 amino acids of human NKG2D preceding the second methionine in the cytoplasmic domain is a highly charged region (isoelectric point pH 12). As our artificial "hNKG2D-S" also lacks this potentially inhibitory sequence, and is still unable to pair with DAP12, other structural elements must explain the difference between human and mouse NKG2D with respect to association with DAP12. By comparison of mouse and human NKG2D (Fig. 2.2), one explanation lies in a 13 amino acid stretch present in mouse, but not human NKG2D, in the cytoplasmic juxtamembrane region. This unique mouse cytoplasmic juxtamembrane sequence (mCJS), designated in Fig. 2.2A, might provide a positive signal for DAP12 association, as this sequence is present in mouse but not human NKG2D. To address this possibility, we created two N-terminal truncations of mouse NKG2D: one that contained the mCJS and another that only contained the transmembrane (TM) and extracellular (EC) domains (mΔNKG2D). Similarly, we created a truncated human NKG2D, which contained only the TM and EC domains (hΔNKG2D) (Fig. 2.2B). Retroviruses encoding mΔNKG2D and hΔNKG2D were used to infect Myc-DAP10 or Flag-DAP12 reporter BaF/3 cells, as described above.

Antibody staining for the appropriate epitope tags and NKG2D revealed that mΔNKG2D was capable of pairing with both DAP10 and DAP12 (Fig. 2.4). This result suggests that the mCJS region is not required for DAP12 association and that the mouse NKG2D TM and EC domains are sufficient for DAP12 association. The same experiment with a truncated mouse NKG2D still containing the mCJS did not

significantly improve DAP12 association, suggesting that this sequence plays no major role in DAP12 association (data not shown). Like "hNKG2D-S", hΔNKG2D was still able to pair with DAP10, but was incapable of pairing with DAP12. This excluded the possibility that the inability of human NKG2D to pair with DAP12 was due to an inhibitory sequence present in the cytoplasmic region. Furthermore, these results suggest that the difference between mouse and human NKG2D, with respect to DAP12 association, must lie within the TM and/or EC domains.

Fig 2.4

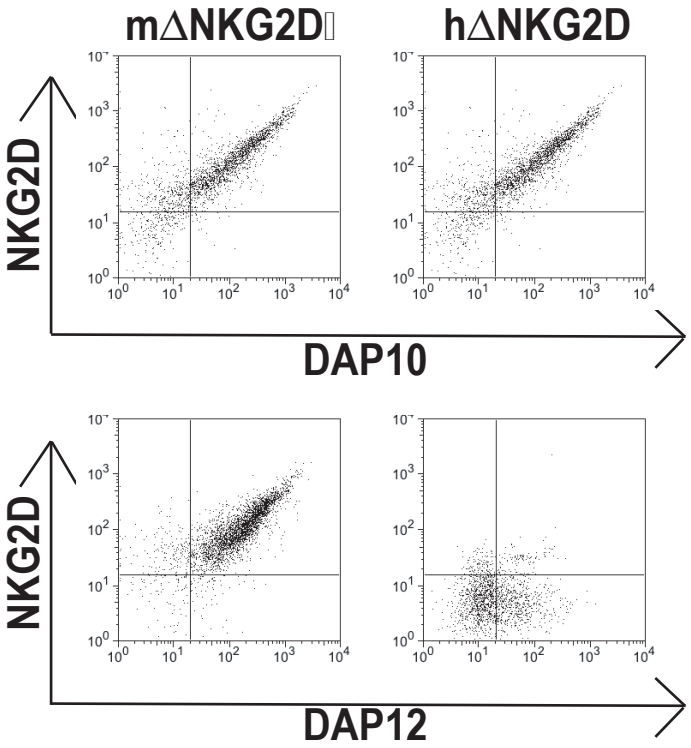

FIGURE 2.4. Mouse NKG2D TM and EC domains are sufficient for DAP12 association.

Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D constructs (described in Fig. 2B) in a vector containing an IRES-GFP element. Samples were stained with the relevant anti-NKG2D and anti-epitope tag mAbs and analyzed by flow cytometry. Results shown are representative of at least three independent experiments.

The mouse NKG2D TM sequence allows human NKG2D to pair with DAP12

Previous studies with a chimeric protein composed of the DAP10 EC - DAP12 TM - DAP10 cytoplasmic (CYT) domains suggested a non-permissive interaction between the transmembrane regions of DAP12 and human NKG2D (75). Furthermore, examination of the protein sequences of human and mouse NKG2D reveals significant sequence divergence within the transmembrane region (Fig, 2.2A, underlined region). These observations spurred the question of whether the difference in DAP12 pairing ability between mouse and human NKG2D could be attributed to their transmembrane (TM) regions. To test the hypothesis that the mouse NKG2D TM is permissive for DAP12 association whereas the human NKG2D TM is not, we created a chimeric NKG2D construct containing the mouse TM domain and human EC domain (mTM hEC) (Fig. 2B) and tested its ability to pair with DAP10 and DAP12 in BaF/3 reporter cells, as previously described. We found that replacing the human TM with the mouse TM region allowed the chimeric protein to stabilize expression of human DAP12 on the cell surface of the transfectants (Fig. 2.5A). In other words, the mouse NKG2D TM permitted the chimeric receptor to associate with DAP12. This conclusion was further supported by the ability to co-immunoprecipitate either DAP10 or DAP12 with the chimeric receptor containing the mouse NKG2D TM region (Fig. 2.5c). By contrast, as reported previously human NKG2D co-immunoprecipitates with DAP10, but not with DAP12 (75, 78). These results suggest a critical difference between the transmembrane domains of human and mouse NKG2D such that human NKG2D is not permissive for DAP12 pairing whereas mouse NKG2D is permissive.

In these experiments, we also tested whether the FQPV motif (Fig. 2.2A boxed sequence) on the extracellular membrane-proximal side of the mouse TM was necessary for DAP12 interaction, as this motif is absent from human NKG2D. Our experiments suggest this motif is unnecessary because chimeric mTM-hEC proteins with or without this sequence associate equivalently with DAP12 (data not shown).

The human NKG2D cytoplasmic domain when associated with the mouse NKG2D TM region is permissive for DAP12 association

Like the mouse NKG2D 13 amino acid "tail", the N-terminal portion of human NKG2D-L contains multiple charged amino acid residues (Fig. 2.2A). Because of this similarity, it is possible that the human cytoplasmic domain behaves like the mouse cytoplasmic domain and may also be repulsive to DAP12 association. Thus, in addition to having a non-permissive TM region in human NKG2D, the repulsive segment present in mouse NKG2D-L cytoplasmic domain might have been conserved in humans to prevent potential association with DAP12. To answer the question of whether the long cytoplasmic domains of human NKG2D or the artificial "short" hNKG2D are permissive for DAP12 binding, we created NKG2D chimeras from the mTM-hEC construct adding back the human "short" intracellular domain (hIC-"Short" mTM hEC), the human long intracellular domain (hIC-Long mTM hEC), or the human "short" intracellular domain with the mouse N-terminal 13 amino acid tail (mTail hIC-"Short" mTM hEC), as portrayed graphically in Fig. 2.2B. As expected, all of these chimeric constructs paired with DAP10 (data not shown).

Using retroviral infection of the Flag-DAP12 reporter BaF/3 cells, we found that the entire human intracellular domain, in both the artificial "short" and natural long isoforms, failed to prevent DAP12 association when the TM region of mNKG2D is present in the chimeric receptors (Fig. 2.5B). In contrast, addition of the mouse “tail” to the human "short" intracellular domain abrogated DAP12 association. These results demonstrate that the N-terminal "tail" sequences of mouse NKG2D is necessary to prevent association of DAP12 with the mouse NKG2D-L isoform. This repulsion cannot be due to charge alone, since the N-terminal segments of both mouse and human NKG2D are rich in acidic and basic amino acid residues. From an evolutionary perspective, mouse NKG2D may have evolved a repulsive tail and alternative splicing of NKG2D as mechanisms to regulate DAP12 adapter signaling. In contrast, as the transmembrane of human NKG2D is not permissive for association with DAP12 it requires no such regulatory region in the cytoplasmic domain and hence this structural feature has not been conserved.

Fig 2.5

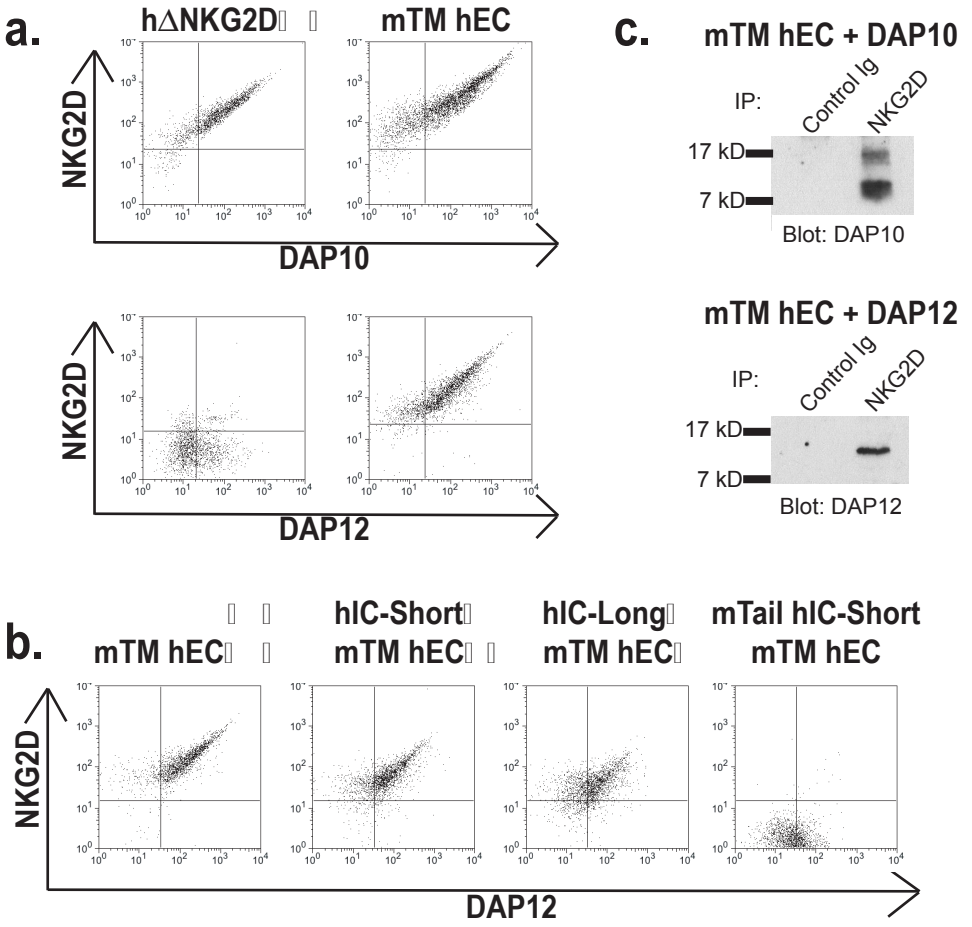

FIGURE 2.5. The mouse NKG2D TM region conveys DAP12 specificity to human NKG2D.

(A,B) Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D constructs (described in Fig. 2B) in a vector containing an IRES-GFP element. Samples were stained with the relevant anti-NKG2D and anti-epitope tag mAbs and analyzed by flow cytometry.

(C) mTM hEC chimeric NKG2D receptor co-immunoprecipitates with DAP10 and DAP12. Transfected mTM hEC reporter cells (panel A) were lysed in Brij-NP-40 lysis buffer. The receptor complexes were immunoprecipitated from lysates with anti-human NKG2D or isotype-matched control mAb. Samples were analyzed by SDS-PAGE and transferred to Immobilon P membrane and probed with goat anti-DAP10 antisera N-17 or anti-DAP12 mAb DX37, followed by HRP-conjugated donkey anti-goat IgG or goat anti-mouse IgG, respectively, and visualized with chemiluminescent substrate. As described previoulsy (Wu, Song et al. 1999), the heterogeneous migration pattern of DAP10 is likely due to O-linked glycosylation of its extracellular domain.

NKG2D TM domains are necessary and sufficient to confer adapter specificity

As the mouse NKG2D truncation (mΔNKG2D) and mTM-hEC chimeric NKG2D proteins paired with DAP12, it was possible that the extracellular portions of human or mouse NKG2D might be necessary for association with DAP12. To address this question and further to ask whether the TM regions of mouse and human NKG2D are sufficient to confer adapter specificity, we created chimeric proteins consisting of the TM domain of either mouse or human NKG2D fused to the EC domain of human CD69 (Fig. 2.6A). CD69 has some structural similarity to NKG2D, as it is also a type II transmembrane-anchored homodimer with an extracellular region consisting of a single C-type-like lectin domain (79-81). However, in contrast to NKG2D, CD69 does not pair with signaling adapters, such as DAP10 or DAP12, and does not possess charged amino acids in its TM region. A truncated form of CD69 consisting of only the TM and EC domains was also constructed as a control, designated ΔCD69 (Fig. 2.6A).

We tested the CD69 chimeras in the DAP10 or DAP12 reporter cells, as previously described, and found that the TM domain of NKG2D was necessary and sufficient to convey adapter specificity to the chimeric proteins (Fig. 2.6B). While the control protein ΔCD69 did not pair with either adapter, the hTM NKG2D-CD69 protein was able to pair with DAP10, but not DAP12. In contrast, the mTM NKG2D-CD69 chimeric protein was able to associate equivalently with either DAP10 or DAP12.

Fig 2.6

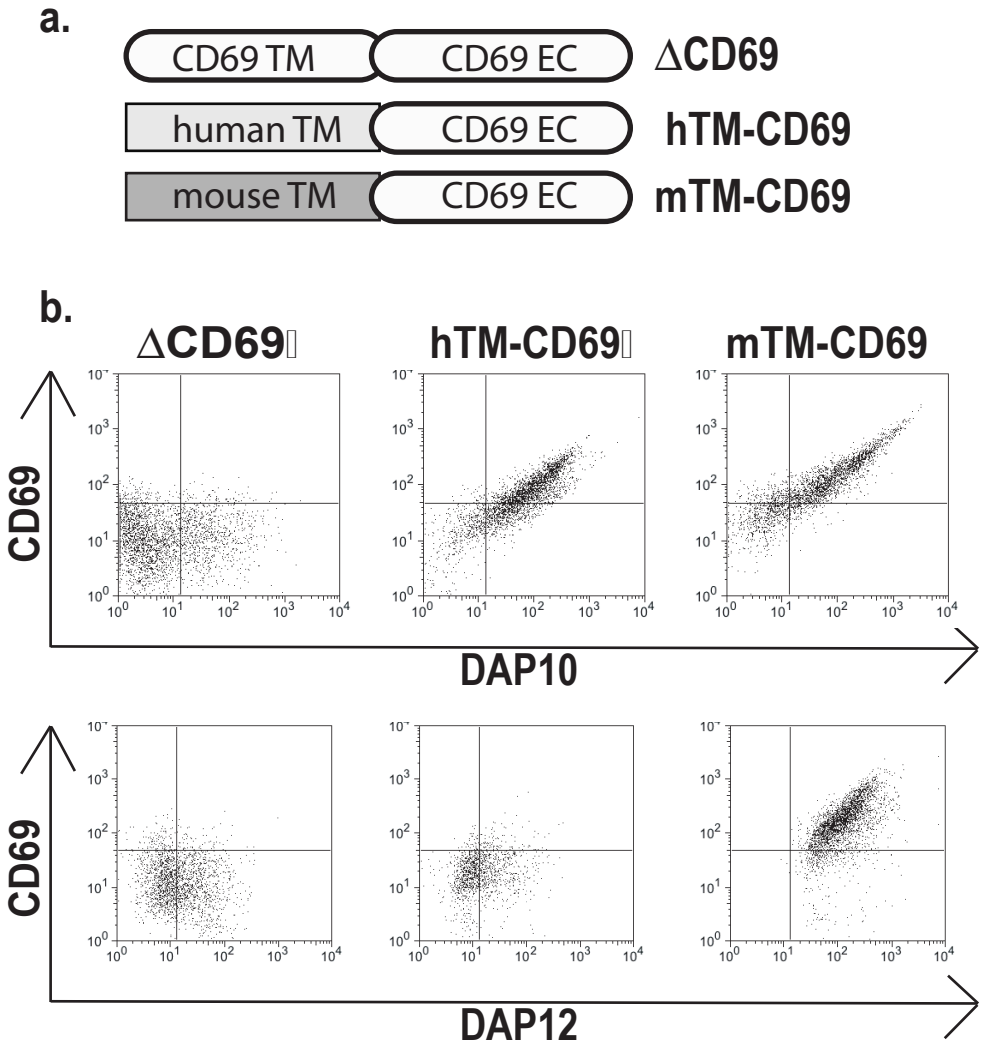

FIGURE 2.6. NKG2D TM regions are necessary and sufficient to confer DAP10 and DAP12 specificity.

(A) Schematic representation of chimeric receptors containing the EC domain of human CD69 and the TM region of human or mouse NKG2D (as described in the Materials and Methods).

(B) Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D TM - CD69 EC constructs in a vector containing an IRES-GFP element. Samples were stained with anti-human CD69 mAb and anti-epitope tag mAbs and analyzed by flow cytometry. Results shown are representative of at least three independent experiments.

We further wished to define requirements for DAP12 association. Closer examination of the mouse TM region revealed a second basic residue present in the mouse but not human TM region. We tested whether this second arginine residue (Fig. 2.2A, boxed sequence) was necessary for DAP12 association by mutating it to an alanine residue via site-directed mutagenesis. We found that an R→A mTM-CD69 mutant still paired with DAP12, indicating that this putative second TM basic residue does not contribute to DAP12 association (data not shown).

These results formally demonstrate that the adapter specificity for human and mouse NKG2D lies exclusively in the TM domain, and this domain is necessary and sufficient to allow adapter pairing. A similar phenomenon has been observed for the receptor specificity of the human DAP10 and DAP12 signaling adapters. Chimeras between DAP10 and DAP12 have illustrated that the receptor specificity of these two adapters also lies in their TM domains (75). Thus, for NKG2D, DAP10, and DAP 12 all of the critical and necessary pairing interactions occur within the transmembrane regions.

Overall, there is little conservation of the TM sequence of human and mouse NKG2D. Only a few amino acids have been conserved, conspicuously the arginine residue in the center of the TM. This extensive diversity in the sequence of the mouse and human NKG2D TM region precludes easy predictions about the critical residues in mouse NKG2D that permit or in human NKG2D prevent association with DAP12.

Discussion

Our results indicate a fundamental structural difference between mouse and human NKG2D. Whereas mouse NKG2D is capable of associating with both DAP10 and DAP12 signaling adapters, human NKG2D is only able to partner with DAP10. We show that this species difference can be mapped to the transmembrane regions of mouse and human NKG2D. As DAP10 signals through a p85 PI3K-mediated AKT pathway (64, 65), and as DAP12 signals through a Syk/Zap-70 mediated ITAM pathway (67), the ability of NKG2D to pair with various combinations of adapters allows it to initiate discreet signaling effects in mice, but not humans. Moreover, direct examination of NKG2D-dependent NK cell activation in DAP12-deficient humans suffering from the Nasu-Hakola disorder demonstrated that the NKG2D receptor functions normally with respect to its ability to induce cytolytic activity in the complete absence of DAP12.

Our findings are consistent with the studies of Billadeau et al. 2003 (22) that demonstrate that a chimeric protein with the cytoplasmic domain of human DAP10 was capable of triggering NK cell-mediated cytotoxicity. However, in these experiments the chimeric DAP10 was introduced into human NK cells expressing a functional DAP12 protein, leaving open the possibility of indirect interactions between the cytoplasmic domains of the chimeric DAP10 and endogenous DAP12 proteins. Our findings provide conclusive evidence for a DAP12-independent role of DAP10 in human NK cell NKG2D-mediated cytotoxicity.

Activated mouse NK cells expressing mNKG2D-S can thus signal through DAP10 or DAP12, whereas resting mouse NK cells and all human NK cells express NKG2D-L and only signal through DAP10. In many cases, DAP10 alone suffices to

initiate cytotoxicity. This is evident from the fact that human NKG2D, which only uses DAP10, stimulates cytotoxicity independent of Syk kinases (69), as well as the evidence that NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases (68). In mice, DAP12 is also independently capable of initiating cytotoxicity through NKG2D-S, as demonstrated by experiments in DAP10-deficient mice (77). Furthermore, mouse NKG2D-S initiated DAP12 signaling contributes to proliferative responses and IFN-γ production (61). A role for DAP10 in IFN-γ production has been suggested using human NK cells and recombinant human NKG2D ligands (65, 78, 82),

It is not completely surprising that the mouse NKG2D transmembrane region can associate with both DAP10 and DAP12 as these two adapter proteins share significant homology. DAP10 and DAP12 share 20% sequence homology (64) and, moreover, their transmembrane domains are very homologous in both sequence (45% homology) and structure (75). In the genomes of both mice and humans, the *DAP10* and *DAP12* genes are adjacent to one another, but in opposite transcriptional orientation (83), a circumstance which likely arose through gene duplication.

Our results illustrate that the transmembrane domain of the immunoreceptor NKG2D provides signaling adapter specificity and accounts for the species difference. Although the human NKG2D TM associates only with DAP10, the mouse NKG2D TM associates with either DAP10 or DAP12. A comparison of NKG2D transmembrane domain sequences across species demonstrates a close relationship between mouse and rat transmembrane sequences (Fig. 2C). Furthermore, the only reported sequence of rat NKG2D lacks an N-terminal "tail" region and is thus structurally similar to mNKG2D-S. Based on the sequence similarity of mouse and rat TM regions and the absence on a

potentially inhibitory "tail" sequence in rat NKG2D that could block DAP12 association, rat NKG2D likely pairs with DAP12. In contrast, other species such as macaque, cow, and pig, display TM sequences more homologous to human NKG2D and may solely signal through the DAP10 adapter protein, although this requires experimental verification.

Although many genes are highly conserved in sequence, expression, and function between mice and humans, *NKG2D* represents a gene that has undergone evolutionary divergence between the two species. Although it acts as an innate immune receptor in both mice and humans, NKG2D demonstrates distinct signaling mechanisms in the two species and none of the human and mouse NKG2D ligands are highly conserved in primary sequence. For example, mice do not possess structural homologs of the human *MICA* and *MICB* genes that are present in the human MHC (84). Furthermore, although the human ULBP genes clearly are functional orthologs of the mouse *Rae-1*, *H60* and *Mult1* genes they have diverged significantly and only demonstrate 15-20% sequence similarity (62). Expression of NKG2D also differs in humans and mice. Whereas all human $CD8^+$ T cells constitutively express NKG2D (26, 55), in mice only activated $CD8^+$ T cells possess NKG2D. Nonetheless, there is evidence that NKG2D is important in immune defense in mice and humans. Viruses and tumors have evolved immune evasion mechanisms to counter the effects of NKG2D (29, 85-87). Thus, perhaps an evolutionary pressure from the host to fight rapidly evolving viral infections and tumors helps explain some of the divergence and distinctions of NKG2D between mice and humans.

Chapter Three

Lectin-Like Transcript 1 is a ligand for NKR-P1A

Abstract

To further understand the biology of NK cells, we decided to study other C-type lectin–like receptors on NK cells. One receptor whose function has remained largely enigmatic is human NKR-P1A (CD161), present on NK and subsets of T cells. Although this molecule was cloned over 10 years ago, no clear function has been assigned to NKR-P1A as its cellular ligand has remained unknown. Here we clearly demonstrate that the C-type lectin LLT1/OCIL is the physiologic ligand for NKR-P1A. Our results illustrate direct and specific binding of LLT1 liposomes to NKR-P1A$^+$ cells. Additionally, we show that LLT1 specifically activates NKR-P1A reporter cells. Moreover, our data indicate that NKR-P1A can inhibit NK cytotoxicity via its interactions with LLT1. The experiments described in this chapter were published in *The Journal of Immunology* (2005) 176: 7796-7799.

Introduction

Rodents have several *Klrb1* (also named *Nkrp1*) genes encoding either activating or inhibitory NK receptors of the C-type lectin-like superfamily, including NKR-P1C -the prototypic NK1.1 antigen defining mouse NK cells in C57BL/6 mice (41). By contrast, only a single, non-polymorphic gene in the Nkrp1 family, designated *KLRB1*, exists in the human genome (40). The human *KLRB1* gene encodes a type II disulfide-linked homodimer, named CD161 or NKR-P1A, which is expressed on most peripheral blood NK cells. Human NKR-P1A is more abundantly expressed on T cells than is mouse NK1.1; human NKR-P1A is on a subset of peripheral T cells, including $CD4^+$ and $CD8^+$ T cells (mostly of the effector/memory phenotype), invariant NKT cells, γδ–TCR^+ T cells, and on a subset of $CD3^+$ thymocytes (40).

Human NKR-P1A is expressed on immature human NK cells, before acquisition of CD16 or CD56 (88), and expression of NKR-P1A can be up-regulated on human mature NK cells by IL-12 (46, 47). NKR-P1A on human memory/effector $CD4^+$ T cells and γδ-TcR^+ T cells (40) has been implicated in the transendothelial migration of these T cells (42, 43). In addition, anti-NKR-P1A monoclonal antibodies (mAb) have been reported to costimulate the anti-CD3 mAb-induced proliferation of human CD1d-specific NK T cells (44), and induce proliferation of immature thymocytes (45). Additionally, cross-linking with an anti-NKR-P1A mAb potently inhibits human NK cell-mediated cytotoxicity against FcR^+ target cells (46, 47).

Recently, ligands for two mouse Klrb1 family members have been identified (48, 49). The activating mouse NKR-P1F receptor recognizes Clr-g (encoded by the *Clec2i* gene) (48), and the inhibitory mouse NKR-P1D recognizes Clr-b (encoded by the *Clec2d*

gene) (49). Another name for Clr-b is osteoclast inhibitory lectin (OCIL), as it was independently identified as an osteoblast-derived glycoprotein, which in soluble form inhibits *in vitro* osteoclastogenesis (50, 51). Although mice have multiple *Clec2* genes, only one ortholog, designated *CLEC2D* (also named lectin-like transcript-1, LLT1), exists in humans. Like mouse Clr-b, human LLT1 was independently identified as an osteoblast-derived protein (designated osteoclast inhibitory lectin, OCIL) that blocks osteoclast differentiation (50, 51). Human *CLEC2D* and the mouse *Clec2* genes are closely genetically linked to the human *KLRB1* gene and the mouse Klrb loci, respectively, in the "NK complex". Moreover, the proteins encoded by the mouse and human Clec2 gene families are type II proteins of the C-type lectin-like superfamily.

The ability of mouse NKR-P1 receptors to associate with Clr ligands prompted the question of whether the same interaction is conserved in humans. In this study, we have examined the ligand specificity of human NKR-P1A and have described the functional consequences of this novel receptor-ligand interaction.

Materials and Methods

Expression and purification of the extracellular domain of the LLT1 protein

The extracellular domain of LLT1 was amplified from human NK cell cDNA using the following primers:

5'GCAAA**GGATCC**A*CATCACCATCACCATCAC*AGAGCTAACTGTCAT 3' and 5'GCAGCG**GAATTC**CTAGACGTGGATATCGGATTTGGAACAAATCCA 3'. The letters in bold indicate restriction enzyme sites and italicized letters encode sequences for His residues. His residues were introduced for purposes of protein purification and engraftment to liposomes. The PCR products were cloned into a pFASTBac vector modified to encode a signal sequence for EGT (ecdysteroid UDP glucosyltransferase) for the efficient production and secretion of type II membrane proteins. The construct was confirmed by DNA sequencing on an ABI 3730 sequencer (Biomolecular Resource facility, JCSMR, ANU) following the manufacturers protocol (Applied Biosystems 2002). The pFASTBac.LLT1 plasmid DNAs was expressed in a baculovirus system using the Bac-to-Bac system (InVitrogen/Life Technologies). The recombinant protein was purified from insect culture supernatants by Ni-NTA affinity chromatography. Purity of the recombinant proteins was assessed by SDS-PAGE.

Liposome binding

The preparation of recombinant LLT1-fluorescent liposome complexes and the procedure for binding to cells was as described previously (89). Briefly, the 6-His rLLT1 protein was bound via the 6-His tag to nitrilotriacetic acid groups embedded in the lipid matrix of fluorescent liposomes, as described (90, 91). Control liposomes were prepared

without 6-His rLLT1. The fluorescent liposome-rLLT1 complexes were added in equal volume to cells prepared in RPMI-1640 medium containing 5 mM Hepes, 10% FCS, and 5% BSA. The cells and liposomes were incubated at RT for 1 h with mixing every 15 min. Cells were washed in PBS containing 5% FCS, fixed in PBS containing 1% paraformaldehyde, and analyzed by flow cytometry. To ensure binding was specific, cells were incubated with an anti-CD161 mAb (10 μg/ml) or an isotype-matched control Ig (10 μg/ml) prior to incubating with the liposome-rLLT1 complexes.

Reporter cell assays

An NFAT-GFP reporter construct was stably transduced into mouse 2B4 T cell hybridoma cells (provided by Dr. H. Arase, Osaka University, Japan). These NFAT GFP reporter cells were transduced with CD3ζ-LLT1 or CD3ζ-NKR-P1A chimeric receptors and analyzed as previously described (92-94). Briefly, reporter and stimulator cells were co-cultured at a 1:4 ratio for 16 h and then analyzed for GFP expression by flow cytometry.

Constructs and transductions

The pMX-s-puro (95) retroviral plasmid was kindly provided by Dr. T. Kitamura (University of Tokyo). Dr. J.P. Houchins (R&D Systems) graciously provided cDNA for human NKp80. cDNA for human CD69, LLT1 with a C-terminal Flag-epitope, human NKR-P1A, human NKp80, and the reporter constructs were subcloned into pMX-s-puro vectors. Chimeric receptors were generated by fusing the extracellular and transmembrane domains of either LLT1 or NKR-P1A to the intracellular domain of human CD3ζ (introduced on the N-terminus of the chimeric receptors). All constructs were confirmed

by DNA sequencing. Plasmid constructs were transfected with Lipofectamine 2000 (InVitrogen) into the Phoenix packaging cell lines (generous gifts from Dr G. Nolan, Stanford University) (74) to produce retroviruses. Retroviruses in medium containing 8 μg/mL polybrene (Sigma) were used to infect IL-3^+ BaF/3 or 2B4 NFAT-GFP reporter cells, as described (92, 93). Infected cells were selected in medium supplemented with 10% FCS, 2 mM L-glutamine, and 1 μg/ml puromycin.

Antibodies

Mouse anti-human NKR-P1A mAbs DX1 and DX12 were produced in our laboratory, and HP3G10 was purchased from MBL International Corporation. FITC-conjugated sheep anti-mouse Ig was purchased from Chemicon. PE-conjugated goat anti-mouse IgG was purchased from Jackson Immunoresearch Laboratories. Cells were incubated with mAb, and subsequently stained with secondary antibodies by incubating on ice for 30 min, and washing 3 times between incubations. Cells were fixed in 1% paraformaldehyde in PBS and analyzed on a FACScan (BD Biosciences), and the data processed using FlowJo software.

Primary cell cultures and cytotoxicity assays

For LLT1-liposome binding studies, venous blood was obtained from healthy volunteers after obtaining informed consent. Polyclonal NK cells were generated from purified peripheral blood NK cells by culture with γ–irradiated MM-170 malignant melanoma cells and recombinant IL-2 as described (96). The *in vitro* cultured cells were exclusively NK cells, as they lacked cell surface CD3ε and expressed CD16 and/or CD56 and/or CD94.

Venous blood was obtained from healthy volunteers after obtaining informed consent, under procedures approved by the Human Ethics Committees of the ACT Department of Health and Community Care and the Australian National University, Canberra, Australia and the UCSF Committee on Human Research. Polyclonal NK cells were generated from PBMC and tested in cytotoxicity assays by methods described previously (72, 96, 97).

Results

LLT1 liposomes bind human NKR-P1A

To test for interactions between human NKR-P1A and LLT1, we generated fluorescent-labeled liposomes containing recombinant human LLT1. We examined LLT1-liposome staining of the mouse Ba/F3 pro-B cell line and BaF/3 cells stably transduced with human NKR-P1A (BaF/3-NKR-P1A). LLT1-liposome complexes bound specifically to BaF/3-NKR-P1A, but not parental BaF/3 (Fig. 3.1A). Whereas LLT1 complexes bound BaF/3-NKR-P1A in the presence of isotype-matched control antibody (solid line), this interaction was specifically blocked by anti-NKR-P1A mAb (dashed line).

As observed with the NKR-P1A transduced Ba/F3 cells, LLT1-liposomes bound to the human NK cell line YT transfected with NKR-P1A (YT-NKR-P1A), but not to untransfected YT cells (Fig. 3.1B, top). The interaction between LLT1-liposomes and YT-NKR-P1A was specifically blocked by all anti-NKR-P1A mAbs tested (i.e. DX1, DX12, and HP3G10), but not by isotype-matched control Ig (Fig. 3.1B, bottom). LLT1-liposomes also recognized endogenous NKR-P1A expressed by *in vitro* activated human NK cells. The binding of LLT1-liposomes to human NK cells was blocked by anti-NKR-P1A mAbs (DX1, DX12, and HP3G10), but not by isotype-matched control Ig (Fig. 3.1C).

Fig 3.1

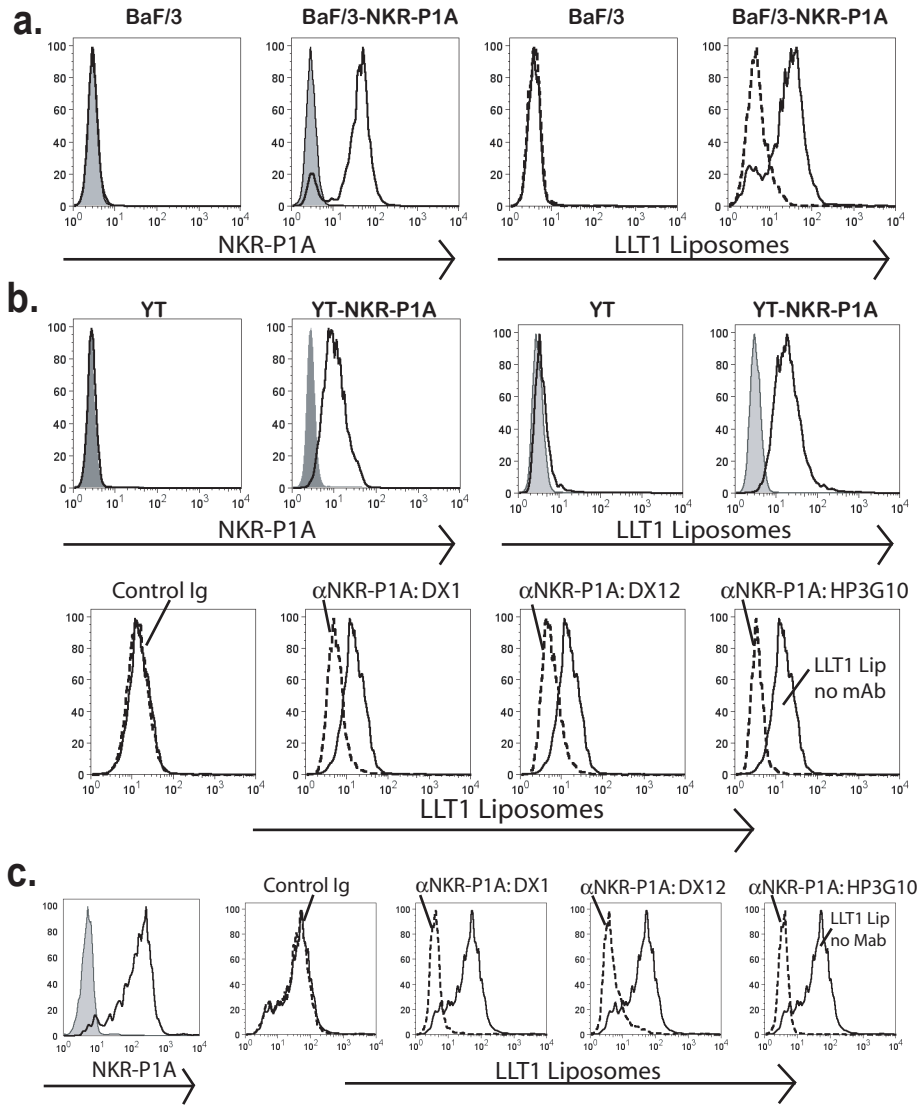

Figure 3.1. Direct binding of LLT1 liposomes to NKR-P1A+ cells.

A, Left: Levels of NKR-P1A on BaF/3 and NKR-P1A-transfected BaF/3 cells (solid: anti-NKR-P1A mAb (clone HP3G10) shaded: control). Right: LLT1 liposomes stain BaF/3-NKR-P1A in the presence of isotype-matched control antibody (solid line) but not in the presence of anti-NKR-P1A mAb (HP3G10) (dashed).

B, Left panels: Levels of NKR-P1A on untransfected YT and NKR-P1A-transfected YT cells (solid line: anti-NKR-P1A mAb (DX1) shaded line: control Ig). Right panels: LLT1 liposomes (solid) stained YT-NKR-P1A but not YT (shaded: unstained cells). Bottom: LLT1 liposome binding to YT-NKR-P1A alone (solid) or in the presence of control Ig or blocking anti-NKR-P1A mAbs, (dashed).

C, Left: NKR-P1A staining of in vitro cultured peripheral blood NK cells (solid: DX1, shaded: isotype-matched control Ig). Right: LLT1 liposome binding to in vitro cultured peripheral blood NK cells alone (solid) or in the presence of control Ig or blocking anti-NKR-P1A mAbs, (dashed).

LLT1 Reporter Cells Recognize NKR-P1A

We created mouse 2B4 NFAT-GFP reporter cells (93) expressing a chimeric protein consisting of the extracellular and transmembrane domains of LLT1 and the intracellular domain of CD3ζ. In this system, conformational changes induced by engaging the CD3ζ-LLT1 chimera result in signaling from the CD3ζ ITAMs, activation of NFAT, and the expression of GFP. Co-culture of the LLT1 reporter cells with NKR-P1A-transfected BaF/3 resulted in robust NFAT-GFP activation, whereas co-culture with parent BaF/3 or BaF/3 expressing the highly related (38% identity) NKp80 (BaF/3-NKp80) did not (Fig. 3.2A). Moreover, the activation of CD3ζ-LLT1 reporter cells by BaF/3-NKR-P1A was specifically blocked by anti-NKR-P1A mAb (DX12), but not by isotype-matched control Ig (Fig. 3.2B). Although the signal transduction capacity of the native LLT1 glycoprotein is unknown, these data indicate that interaction with NKR-P1A induces a conformational change through the transmembrane and ectodomain of LLT1, resulting in activation of NFAT through the CD3ζ-cytoplasmic domain in the chimeric receptor.

NKR-P1A Reporter Cells Recognize LLT1

Similarly, we created 2B4 NFAT-GFP reporter cells expressing a CD3ζ-NKR-P1A chimera and tested the ability of these cells to recognize LLT1-bearing cells. NKR-P1A reporter cells expressed GFP when co-cultured with LLT1-transduced BaF/3 (BaF/3-LLT1), but not with untransfected BaF/3 or BaF/3 expressing the highly related (38% identity) human CD69 glycoprotein (BaF/3-CD69) (Fig. 3.2C). Furthermore,

LLT1-induced activation of the NKR-P1A reporter cells was specifically inhibited by co-incubation with anti-NKR-P1A mAbs, but not isotype-matched control Ig (Fig. 3.2D).

Fig 3.2

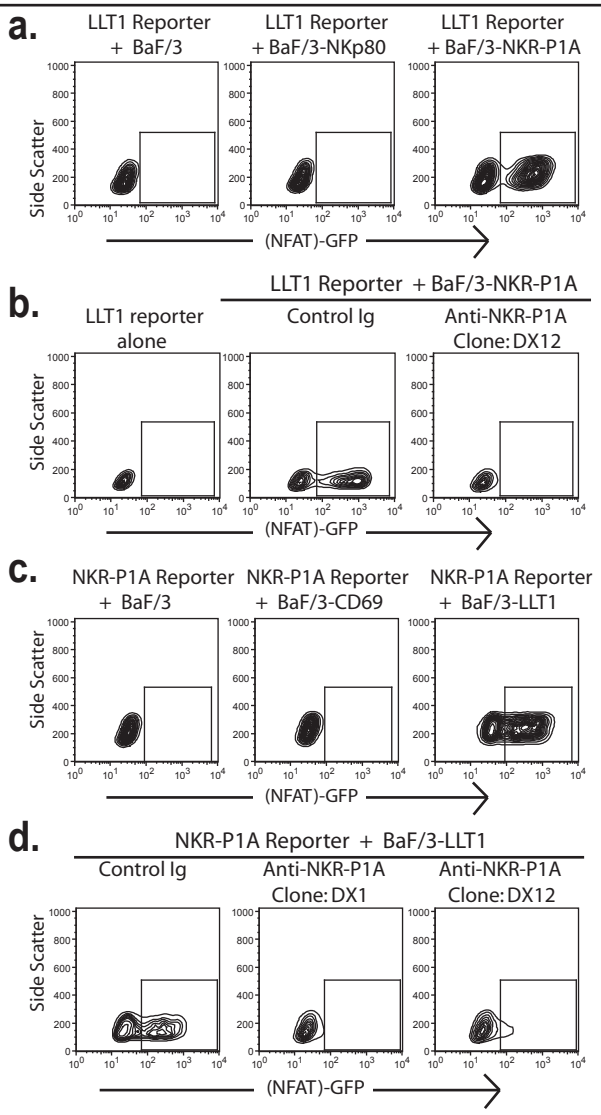

Figure 3.2. LLT1 and NKR-P1A reporter cells recognize NKR-P1A and LLT1, respectively.

A, NKR-P1A-transfected BaF/3 (BaF/3-NKR-P1A), but not parent BaF/3 or BaF/3 expressing human NKp80 (BaF/3-NKp80), induced NFAT (as readout by GFP production) when co-cultured with the CD3z-LLT1 chimera NFAT-GFP reporter cells.

B, Activation of CD3z-LLT1chimera reporter cells by BaF/3-NKR-P1A was specifically blocked by anti-NKR-P1A mAb (DX12).

C, LLT1-transfected BaF/3 (BaF/3-LLT1), but not parental BaF/3 or BaF/3 expressing human CD69, induced NFAT (as readout by GFP production) when co-cultured with CD3z-NKR-P1A chimera NFAT-GFP reporter cells.

D, Activation of CD3z-NKR-P1A chimera reporter cells by BaF/3-LLT1 was specifically blocked by anti-NKR-P1A mAbs (DX1 and DX12).

LLT1 inhibits NK cytotoxicity via interactions with NKR-P1A

Prior studies have demonstrated that NKR-P1A inhibits NK cell cytotoxicity (40). Now with a defined ligand for NKR-P1A, we examined the function of NKR-P1A in NK cells using target cells expressing its physiological ligand. We found that EBV-transformed 721.221 B lymphoblastoid cells express LLT1 mRNA, as detected by PCR (data not shown). In accordance, we found that 721.221 activate CD3ζ-NKR-P1A reporter cells, an effect that was specifically blocked by anti-NKR-P1A mAb, but not isotype-matched control Ig (Fig.3.3A).

We tested the cytolytic ability of YT cells and YT cells expressing NKR-P1A (YT-NKR-P1A) against 721.221 targets. NKR-P1A-transfected YT cells had diminished cytotoxic capacity against 721.221 targets as compared to untransfected YT cells (Fig. 3.3B); equivalent killing by YT and YT-NKR-P1A was seen against human CD48-transduced BaF/3 target cells, indicating that both YT and YT-NKR-P1A have similar lytic potential (data not shown). Furthermore, the NKR-P1A-mediated inhibition of 721.221 lysis seen in YT-NKR-P1A cells was reversed by anti-NKR-P1A $F(ab')_2$, but not isotype-matched control $F(ab')_2$ (Fig. 3.3C).

NK cells can be stimulated through several activating receptors that use biochemically distinct signaling pathways, for example human NKG2D, which recognizes the MICA ligand, and human CD244, which recognizes the CD48 ligand (7). We examined the effects of NKR-P1A on these activation pathways by transducing LLT1 into BaF/3 target cells expressing MICA or CD48 and determining the effect of NKR-P1A engagement on NK cell-mediated cytotoxicity. We tested the ability of LLT1 to modulate peripheral blood NK cell lysis of BaF/3 target cells expressing the NK

activating ligands CD48 or MICA. LLT1 expression by these target cells diminished NK cell-mediated cytotoxicity induced by either the CD48/CD244 pathway (Fig 3.3D, left) or the MICA/NKG2D pathway (Fig 3D, right), an effect which was mediated through interactions with NKR-P1A and was specifically reversed by anti-NKR-P1A $F(ab')_2$, but not isotype-matched control $F(ab')_2$ (Fig. 3.3D).

Fig 3.3

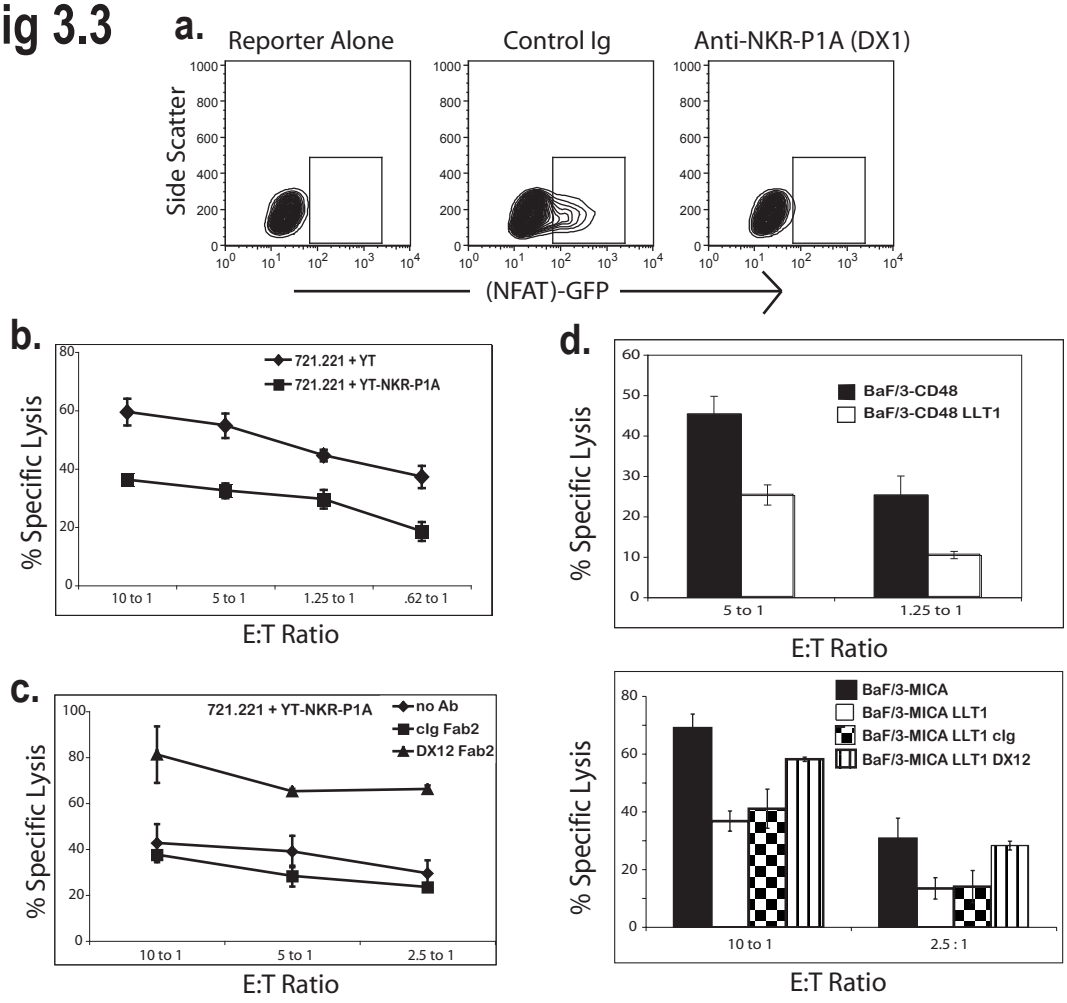

Figure 3.3. LLT1 inhibits NK cell-mediated cytotoxicity via interactions with NKR-P1A.

A, 721.221 cells activated NKR-P1A-CD3z chimera reporter cells, which was blocked by anti-NKR-P1A mAb (DX1).

B, NKR-P1A-transfected YT cells have diminished cytotoxic capacity against 721.221 targets as compared to untransfected YT cells.

C, NKR-P1A-mediated inhibition of 721.221 lysis was reversed by anti-NKR-P1A F(ab')2, but not isotype-matched control F(ab')2. F(ab')2 was used at 10 mg/ml.

D, In vitro cultured peripheral blood NK cell-mediated cytotoxicity against BaF/3-CD48 was reduced by LLT1 expression (left). LLT1-induced inhibition of NK cell-mediated cytotoxicity against Ba/F3-MICA target cells was reversed by anti-NKR-P1A F(ab')2 but not isotype-matched control F(ab')2 (right).

Discussion

These data prove that LLT1 is a ligand for human NKR-P1A and this interaction inhibits NK cytotoxicity. In mice, the LLT homolog Clr-b interacts with inhibitory receptor NKR-P1D. Interestingly, expression of Clr-b, like MHC class I, is widely distributed (49). Recently, potential orthologs for NKR-P1A and LLT1, *B-NK* and *B-lec,* respectively, have been described in the MHC region in chickens (98). This raises the questions of whether *B-NK* and *B-lec* interact, and whether NKR-P1A – LLT1 – like interactions have been preserved through evolution. LLT1 has been reported to be transcribed in T cells, B cells, and NK cells (99), where its expression can also be induced by PMA (100), and in osteoblast cell lines, where its expression is induced by IL-1α (93). Like LLT1, *B-lec* expression is also induced by PMA treatment (98). Collectively, our findings provide another example of a functional interaction between two genomically linked C-type lectin proteins encoded by genes in the "NK complex". This clearly raises the question of whether other C-type lectins within this genomic region are interacting, and what the functional consequences may be. Additionally, the existence of the LLT1 – NKR-P1A interaction in humans provides yet another mechanism for the fine-tuning of NK cell and T cell responses using an inhibitory receptor that recognizes a non-MHC ligand.

Chapter Four

Induction of LLT1, a ligand of human NKR-P1A (CD161), on B cells and Dendritic cells

Abstract

Lectin Like Transcript-1 (LLT1) (also named osteoclast inhibitory lectin, OCIL) is a physiologic ligand for the human NKR-P1A (CD161) receptor, present on natural killer (NK) cells and T cells. On NK cells interaction of LLT1 with NKR-P1A inhibits cytotoxic function. To further understand the physiological relevance of this interaction, we developed monoclonal antibodies against LLT1, examined the expression of LLT1, and explored the role of NKR-P1A on T cells. We show that LLT1 is expressed on activated B cells and is strongly induced by the TLR9 agonist, CpG DNA. We further demonstrate that stimulation of blood lymphocytes via TLR3, TLR4, TLR7, TLR8 or TLR9 all induce LLT1 and that LLT1 is expressed in activated plasmacytoid dendritic cells and monocyte-derived dendritic cells.. Lastly, we illustrate that although NKR-P1A inhibits NK cell activity and can inhibit $CD8^+$ T cell TNFα production, NKR-P1A failed to inhibit or augment TcR-dependent activation of NKR-P1A-bearing $CD4^+$ T cells. The experiments described in this chapter are currently being prepared for publication.

Introduction

Human NKR-P1A (CD161), encoded by the *KLRB1* gene, represents the only human relative of the rodent NKR-P1 family, which includes NKR-P1C, the prototypic NK1.1 antigen defining mouse NK cells in C57BL/6 mice (41). It is a member of the C-type lectin superfamily and the protein is a type II disulfide-linked homodimer (40). Human NKR-P1A is expressed on immature human NK cells, before acquisition of CD16 or CD56 (88), and expression of NKR-P1A can be up-regulated on human mature NK cells by IL-12 (46, 47). NKR-P1A is also expressed on human memory/effector $CD4^+$ T cells, $CD8^+$ T cells, γδ-TcR^+ T cells, and ~60% of invariant NKT cells, as well as a subset of $CD3^+$ thymocytes (40, 101, 102). Experiments examining the role of NKR-P1A on T cells have suggested a stimulatory role for this receptor: anti-NKR-P1A monoclonal antibodies (mAb) costimulated the anti-CD3 mAb-induced proliferation of human CD1d-specific NK T cells (44), and induced proliferation of immature thymocytes (45). In contrast, cross-linking with an anti-NKR-P1A mAb potently inhibits human NK cell-mediated cytotoxicity against FcR^+ target cells (46, 47).

We and others identified Lectin-Like Transcript-1 (LLT1), also known as osteoclast inhibitory lectin, OCIL, as a physiologic ligand for NKR-P1A (10, 103). Interestingly, the gene encoding LLT1, *CLEC2D*, lies directly adjacent to the *KLRB1 gene* in the NK genomic complex, located on human chromosome 12 (104). Recently, the closest homolog to LLT1, Activation Induced C-Type Lectin (AICL), and the closest homolog to NKR-P1A, NKp80, were shown to interact, providing another example of genomically linked C-type lectins recognizing one another (105). Functional studies indicated that the interaction of LLT1 with NKR-P1A serves to inhibit NK cytotoxicity

(10, 103). To further investigate the functional significance of the LLT1-NKR-P1A interaction, we developed monoclonal antibodies and a polyclonal rabbit serum against LLT1. We demonstrate that LLT1 is expressed on many B cell lines and on activated B cells. As CpG DNA stimulation gave the strongest LLT1 induction on B cells, we examined LLT expression following treatment with a panel of TLR ligands and found that TLR3, TLR4, TLR7, TLR7 8 & TLR9 stimulations all induced LLT1. TLR activated plasmacytoid dendritic cells and TLR activated monocyte-derived dendritic cells both expressed LLT1. Lastly, we examined the function of NKR-P1A on T cells and found that whereas NKR-P1A inhibits degranulation and cytokine production in NK cells, NKR-P1A did not modulate TcR-induced cytokine production in $CD4^+$ T cells or degranulation in $CD8^+$ cells, but did inhibit TNFα production in $CD8^+$ T cells.

Materials and Methods

Constructs and transductions

The pMX-s-puro (95) retroviral plasmid was kindly provided by Dr. T. Kitamura (University of Tokyo). AICL cDNA was generously provided by (Dr. Jorg Hamann). AICL and LLT1 with a C-terminal V5 epitope tag were subcloned into pMX-s-puro and transfected with Lipofectamine 2000 (InVitrogen) into the Phoenix packaging cell lines (generous gifts from Dr G. Nolan, Stanford University) (74) to produce retroviruses. Retroviruses in medium containing 8 μg/mL polybrene (Sigma) were used to infect IL-3$^+$ BaF/3 (generously provided by Dr. S. Tangye) or 721.221 cells, as described (92, 93). Infected cells were selected in medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin and streptomycin, and 1 μg/ml puromycin.

Antibodies and staining

Mouse anti-human NKR-P1A (CD161) mAbs DX1 and DX12 were produced in our laboratory, and B199.2 was purchased from GeneTex. PE-conjugated goat anti-mouse IgG was purchased from Jackson Immunoresearch Laboratories, streptavidin secondary reagents were from BD. FITC-conjugated anti-CD107a, PE-conjugated anti-CD56, PerCP-conjugated anti-CD4, PE-Cy7-conjugated CD8, APC-conjugated anti-CD161 DX12, PE-conjugated anti-IL-2, APC-conjugated anti-IFNγ, Alexa700-conjugated anti-TNFα, and intracellular staining reagents were from BD Pharmingen

To produce mouse anti-human LLT1 mAbs (clones 402624 and 402659, both IgG1 isotype antibodies, (R&D Systems) mice were immunized with E. coli-expressed recombinant human LLT1 extracellular domain (amino acids 57-191; Accession number

NP_037401). Hybridoma supernatants were screened in a direct ELISA for their ability to detect the recombinant LLT1. Positive wells were subsequently screened in a cell ELISA for recognition of an LLT1 P815 transfectant but not an irrelevant transfectant. Hybridomas showing the desired reactivity were subcloned by limiting dilution and further screened on LLT-positive B cell lines 721.221 and Raji. Antibody was purified from hybridoma supernatants on Protein G columns.

Rabbit Anti-LLT1 antisera was made by immunizing rabbits with a fusion protein of Glutathione *S* Transferase (GST) and the intracellular domain of LLT1 (amino acids 1-33). Antibodies were affinity purified on a fusion protein of Mannose Binding Protein (MBP) and the intracellular domain of LLT1 covalently attached to CNBr-activated Sepharose (Sigma).

Cells were incubated on ice for 30 min with mAb, washed, and subsequently stained with secondary antibodies. Cells were fixed in 1% paraformaldehyde in PBS and analyzed on a FACScan (BD Biosciences). The data were processed by using FlowJo software (TreeStar).

Biochemistry & Immunoblots

SDS-PAGE and immunoblots were performed as described (92). Neuraminidase (Roche) and PNGase F (New England BioLabs) were used according to the manufacturer's instructions. For immunoblots, anti-LLT1 affinity-purified antibodies were used at 1 μg/mL and detected with HRP-conjugated goat-anti-rabbit IgG (Jackson Immunoresearch)

Primary cell cultures

Venous blood was obtained from healthy volunteers after obtaining informed consent, under procedures approved by the UCSF Committee on Human Research. Small, resting memory T cells were isolated as follows: Percoll density gradient centrifugation of peripheral blood mononuclear cells (PBMC) yielded a low-buoyant density leukocyte interface and a small, resting peripheral blood lymphocyte (PBL) pellet, which was then depleted of non-T cells and naïve T cells with antibodies against CD14, CD16, CD19, CD56, CD45RA, CD123, and CD236. Small, resting memory T cells were cultured in RPMI-1640 medium, 10% FCS, 2 mM glutamine, penicillin and streptomycin, (complete medium) and 200 U/ml human recombinant IL-2 (provided by the NCI BRB Preclinical Repository). For NK assays, the low-buoyant density cells from the Percoll interface layer were isolated and grown in complete medium with 200 U/ml IL-2. Experiments were performed after at least 24-hour culture in IL-2.

Monocyte-Derived Dendritic (Mo-DC) cells were generated as follows: $CD14^+$ monocytes were positively selected from PBMC using magnetic beads (Miltenyi Biotech) and cultured for 6 days in the presence of 25 ng/mL IL-4 and 50 ng/mL GM-CSF (R&D Systems) in complete RPMI. Cytokines were replenished on days 2 and 4. Cells were harvested on day 6 and verified to be $CD14^-$, $CD1\square^+$, $CD11c^+$ and $HLA\text{-}DR^+$.

B cells were isolated from PBMC by using anti-CD19 mAb-conjugated magnetic beads (Miltenyi Biotec) and grown in Isocove's DMEM supplemented with 10% FCS, 2 mM glutamine, penicillin and streptomycin, and 10 U/mL IL-2.

Human peripheral pDCs were positively selected from PBMC with anti-BDCA-4-coated microbeads and sorted by flow cytometry as CD3-CD4+CD8-CD11c-CD14-

CD16-CD19-CD56-. pDCs were cultured at $1x10^6$ cells per ml in RPMI 1640 media supplemented with 10% heat-inactivated FBS in the presence of various stimuli: 20 μg/ml IL-3, 5 μM CpG A (2216), 5 μM CpG B (2006), 5 μM CpG C (C274), heat-inactivated HSV (MOI 10), influenza virus (MOI 10) or 0.1μg/ml R848. After 20 hrs, total RNA was obtained and reverse-transcription was performed as described (106). For protein expression analysis, cells activated for 48 hrs then harvested and lysed.

For microarray experiments, human peripheral B cells were positively selected from PBMC with anti-CD19-coated microbeads and sorted by flow cytometry as CD3-CD4-CD8-CD11c-CD14-CD16-CD20+CD56-BDCA2-. B cells were cultured at $1x10^6$ cells per ml for 20 hrs in RPMI 1640 media supplemented with 10% heat-inactivated FBS in the presence of 5 μM CpG B (2006) or IL-2 (50 U/ml)/IL-10 (100 U/ml). Memory B cells were positively selected from PBMC with anti-CD19-coated microbeads and sorted by flow cytometry as CD3-CD4-CD8-CD11c-CD14-CD16-CD20+CD27+CD56-BDCA2-.

Microarray Data

A gene expression database, which included the major human immune cell types in peripheral blood, was established as described (106). The Positional Dependent Nearest Neighbor model (107) was used to estimate the gene expression values from the probe intensity values. The final expression output was normalized with the numerical value of one representing the estimated threshold of basal expression.

Quantitative RT-PCR

Sense *ATTACACCATCTGAATTGCCTGC* and antisense *GCGCCAAATTAAGGTAGCTTTAATA* primers were used with probe *ACCCAGGTTGTCTGCATTCAAAAGAGCA* to detect LLT1 transcripts by using an ABI 7300 Real Time PCR System. LLT1 transcript levels are shown relative to controls; GAPDH for pDC, PBMC Donor A and B cells, or S18 for PBMC Donor B.

Cellular Assays

For mAb crosslinking studies, B cells were cultured for 48 hours on 24-well tissue culture plates coated with DOTAP and mAbs, as described (93). For other stimulations, low buoyant density lymphocytes, monocyte-derived dendritic cells (Mo-DC) and B cells were treated for 48 hours with 1 μg/mL E. coli LPS (Sigma), 5 μM CpG-B-2006 (Operon) for B cells, 2 μM each CpG-A,B,C (Operon) for other cells, 50 μg/mL poly I:C (Amersham Pharmacia), 1-5 μg/mL R837 (Invivogen), 5 μg/mL CL-075 (Invivogen), 5000 U/mL IFNα2b Intron A (generously provided by Larry Fong, UCSF), 10 μg/mL Zymosan (Sigma), Peptidoglycan 10 μg/mL (Invivogen), 1 ng/mL IL-1α (R&D Systems), or 15 ng/mL PMA (Sigma).

T cells (1.5×10^5 / well) in 200 μl of RPMI-1640 with 10% FCS, 2 mM glutamine, penicillin, and streptomycin were co-cultured in 96-well round-bottom plates with irradiated (3000 rad) mouse P815 cells (2×10^4 / well) and stimulating mAbs for 6 hours to induce the production of cytokines, in the presence of Golgiplug and Golgistop (BD Pharmingen). For P815 stimulations, 0.5 μg/mL anti-CD3 (clone Leu4) and 0.5

μg/mL anti-CD28 (clone L293) were used for suboptimal stimulation and anti-NKR-P1A or an isotype-matched control IgG was added at 0.25 μg/well. For T cell degranulation assays, cells were cocultured with human CD80-transfected P815 cells (P815-B7.1) (108) and 0.5 μg/mL anti-CD3 for 2 hours, stained with FITC-conjugated anti-CD107a and antibodies against CD4, CD8, and CD161, and then analyzed on an LSRII flow cytometer (BD). For cytokine assays, cells were cultured for 6 hours, as described above, stained for surface proteins CD4, CD8, and CD161, and then permeabilized and stained for intracellular cytokines according to the manufacturer's instructions. Stimulations with Raji cells were as follows: 2.5 ng/mL SEB (Toxin Technologies) was used to stimulate T cells ($1.5x10^6$ / well) cocultured with Raji cells ($0.25 x10^6$ / well) in the presence of Golgiplug with or without blocking antibodies (0.5 μg /well) for 6 hours, and then stained as above.

For NK cell assays, low-buoyant density cells ($1.5 x 10^5$ / well) in 200 μl of complete medium were co-cultured in 96-well round-bottom plates with irradiated (3000 rad) mouse P815 cells ($1.5 x 10^4$ / well). For P815 experiments, cells were stimulated with 2.5 μg/mL anti-2B4 (clone C1.7) and 2.5 μg/mL of either isotype-matched control Ig or anti-NKR-P1A mAb for 2.5 hours in the presence of Golgiplug and Golgistop (BD Pharmingen), were stained with mAbs against FITC-conjugated anti-CD107a, PE-conjugated anti-CD56 (BD), and PE-Cy5-conjugated anti-CD3, and then analyzed by flow cytometry. For Raji experiments, low-buoyant density cells ($1.5 x 10^5$ / well) were co-cultured with Raji cells ($2 x 10^4$ / well) for 6 hours with Golgiplug and Golgistop alone or in the presence of, isotype-matched control F(ab)'2 or blocking anti-NKR-P1A $F(ab)'_2$

, were stained with CD107a, CD56 and CD3 mAbs, and then stained for cytokines as described above.

Results

Human LLT1 is a disulfide-bonded, glycosylated homodimer

In order to understand the functional role of an NKR-P1A and LLT1 interaction, we first sought to discover under what conditions and in which cell types LLT1 is expressed. To accomplish this, we created mAbs (clones 402624 and 402659) against the extracellular domain of LLT1. Mice were immunized with the extracellular domain of LLT1 in soluble recombinant form, and then boosted with injection of mouse BaF/3 cells expressing human LLT1. Hybridomas were screened for reactivity against the soluble recombinant protein, and for reactivity against BaF/3 cells expressing LLT1. We also characterized and used a recently commercially available antibody, clone 4C7, against LLT1. These mAbs specifically recognized human LLT1-transfected BaF/3 cells, but did not recognize parental untransfected BaF/3 or BaF/3 expressing human AICL, the closest homolog of LLT1 (Fig. 4.1a). We tested whether the new anti-LLT1 mAbs (clones 4C7 and 402624 & 402659) were capable of blocking the interaction with NKR-P1A by using our previously described NKR-P1A reporter cells and found these mAbs lacked blocking activity (data not shown).

We also created an affinity-purified rabbit antiserum against the intracellular domain of LLT1 (LLTic) for immunoblots. We immunized rabbits with a Glutathione-*S*-Transferase-LLTic chimeric protein and used a Mannose Binding Protein-LLTic chimeric protein to purify LLTic-reactive antibodies. This anti-LLT1 antiserum, but not pre-immune control serum, specifically recognized LLT1-transfected BaF/3 cells, but not untransfected BaF/3 or AICL-transfected BaF/3 (Fig. 4.1b,c). SDS-PAGE analysis

revealed that LLT1 migrated at about 65 kD in its non-reduced form. When lysates were treated with neuraminidase and Protein N-Glycanase F (PNGase), a significant shift in migration was observed, indicating the presence of N-linked glycans on LLT1 (Fig. 1d). Reduced monomers of LLT1 migrated at approximately 35 kD or at 25 kD when treated with neuraminidase and PNGase, which is close to the predicted 22 kD molecular weight of the LLT1 polypeptide.

Fig 4.1

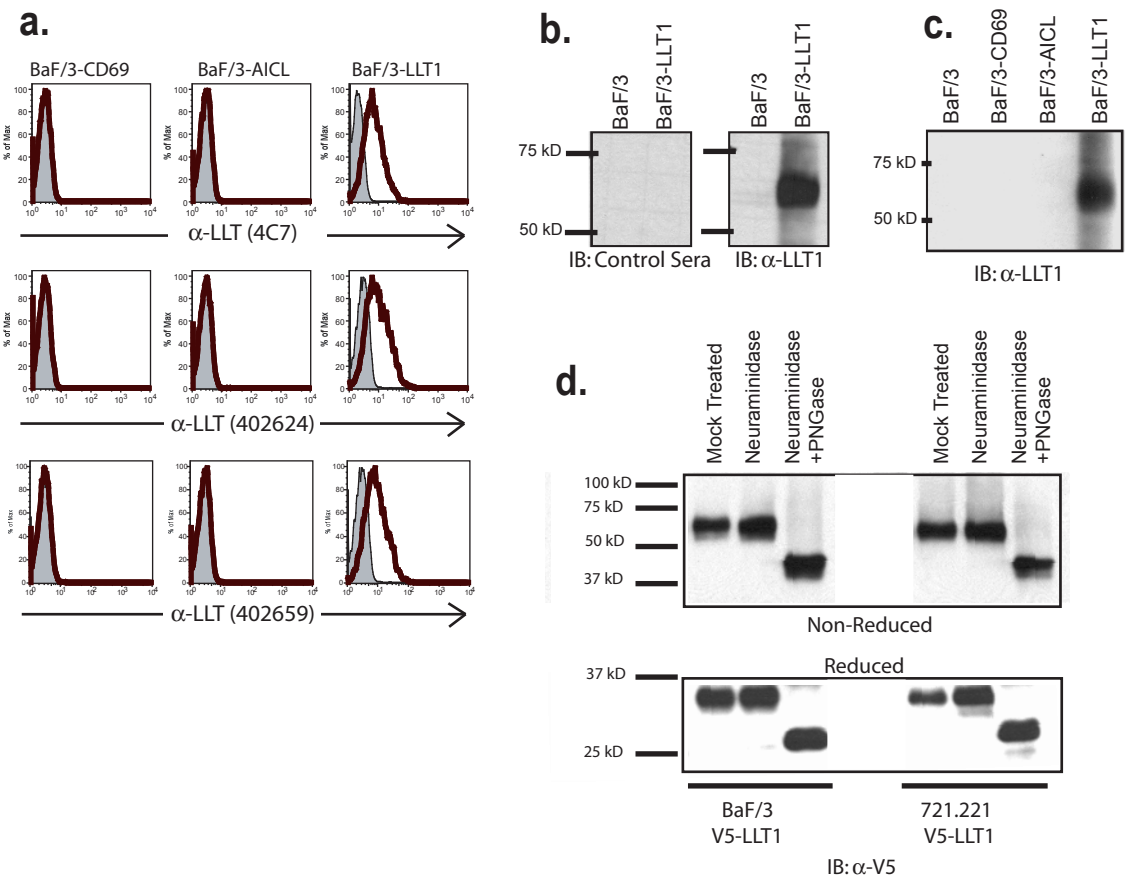

Figure 4.1. LLT1 is a glycosylated disulfide-bonded homodimer.

A, Monoclonal antibodies against LLT1 specifically recognize LLT1-transfected cells but not untransfected cells or cells transfected with related protein, AICL.

B, Anti-LLT1 antisera but not control pre-immune sera specifically detects LLT1.

C, Protein N Glycanase F (PNGase) treatment of LLT1 transfectants reveals that LLT1 is an N-glycosylated dimer.

LLT1 is expressed on activated B cells

We had previously detected LLT1 on the EBV-transformed B cell line 721.221 and reported that LLT1 on this target cells inhibited NK cell-mediated cytotoxicity through interactions with NKR-P1A on NK cells (10). We examined a panel of human B cell lines for LLT1 expression and detected LLT1 protein on a number of B cell lines, including Namalwa, Daudi, Raji, Ramos, RL, and RPMI-8866 (Fig. 4.2a). Additionally, these cell lines all expressed LLT1 mRNA by RT-PCR and demonstrated LLT1 protein by Western blot analysis (data not shown). Interestingly, out of the five Burkitt's lymphoma cell lines analyzed (BJAB, Namalwa, Daudi, Raji, and Ramos), four expressed LLT1. There was no correlation, however, between LLT1 expression and Epstein Bar Virus transformation status.

In examining whether primary B cells express LLT1, we performed microarray expression experiments which indicated that whereas both freshly isolated naive B cells and memory B cells were negative for LLT1 mRNA, CpG-activated B cells expressed LLT1 (Fig. 4.2b). We also detected increased LLT1 mRNA transcripts in B cells by quantitative RT-PCR (Fig. 4.2c). To confirm this was a direct effect of CpG on B cells, to exclude the requirement of other cell types, and to determine whether other methods of activating B cells can induce LLT1, we examined LLT1 expression in purified B cells. B cells isolated from PBMC were treated with plate-bound antibodies against CD40 or IgM or were stimulated with CpG for 48 hours and then lysed for examination by Western blot. Whereas resting peripheral blood B cells expressed very low amounts of LLT1, stimulation via CD40 or IgM crosslinking induced LLT1 protein, as did CpG DNA treatment (Fig. 4.2d). Treatment with CpG DNA consistently resulted in the highest

induction of LLT1 expression. These data indicated that IgM, CD40, or CpG stimulation of B cells is sufficient to induce LLT1 expression without a requirement for other cell types.

To examine the specificity of CpG DNA stimulation, we treated PBMC with a panel of TLR stimuli and examined B cell expression of LLT1. We detected LLT1 on CpG-treated B cells, but not on B cells treated with other TLR stimuli (Fig. 4.2e). Of note, human B cells can express TLR1, TLR2, TLR4, TLR7, TLR9, and TLR10 (109, 110).

Fig 4.2

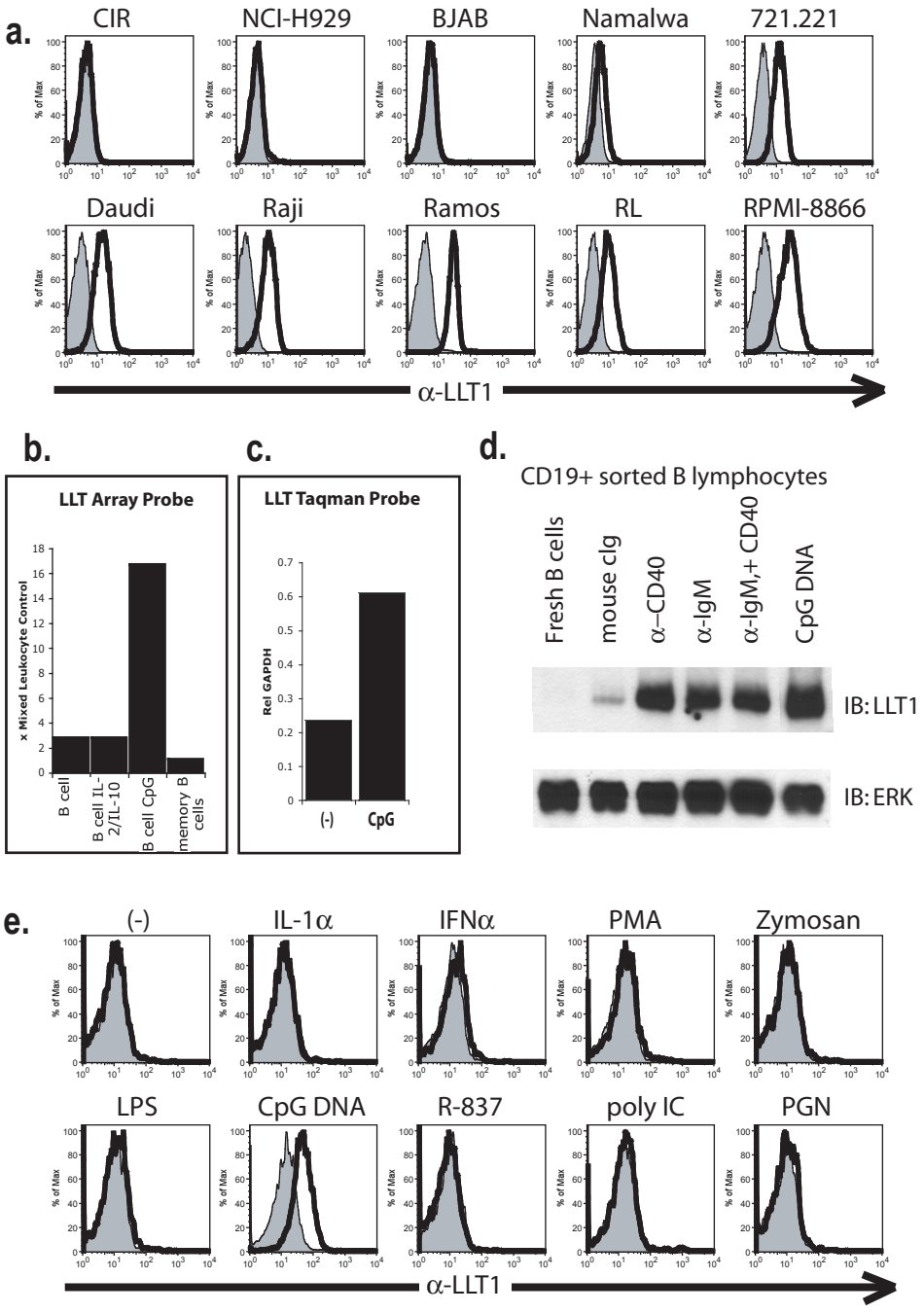

Figure 4.2 Activated B cells express LLT1.

A, Many immortalized B cell lines express LLT1

B, Microarray expression data reveals that B cells treated with CpG, but not resting or memory B cells, express LLT1.

C, Quantitative RT-PCR confirms that CpG induces LLT1 expression in B cells.

D, Western blot of purified B cells demonstrates that activation through CD40, IgM or TLR9 is sufficient to induce LLT1 protein in sorted B cells.

E, CpG DNA, but not other TLR stimuli, induces LLT1 protein on the surface of B cells.

Stimulation through TLR3, TLR4, TLR7, TLR7 & TLR9 induce LLT1

Previous work suggested that LLT1 mRNA is expressed in osteoblast cell lines and that LLT1 transcript levels increase with IL-1α treatment (51). Data also exist that peripheral blood mononuclear cells (PBMC) express LLT1 mRNA when treated with phorbol-12-myristate-13-acetate (PMA) (52). After seeing LLT induced on CpG treated B cells, we examined whether other Toll-Like Receptor (TLR) stimulations might induce LLT1 expression. To accomplish this, we treated low buoyant density peripheral blood lymphocytes with various TLR stimuli for 48 hours then tested for LLT1 protein by Western immunoblots. We saw that TLR9 lignad CpG DNA induced LLT1 expression, as did TLR7 ligand R-837 and TLR8 lignad CL-075, and to a lesser extent TLR3 ligand poly I:C and TLR4 ligand LPS (Fig 4.3). IFNa2b also induced LLT1 expression however TLR2 ligands peptidoglycan and Zymosan did not induce LLT1 (Fig 4.3).

Although other groups have reported LLT1 expression on monocytes and NK cells (111), we examined resting monocytes, resting and IL-2 activated NK cells, and resting and IL-2 activated T cells and found no evidence for LLT protein by flow cytometry and Western blot analysis (data not shown). We did, however, see LLT expression on NK cells lines NKL and YT2C2, suggesting it may be possible for NK cells to express this protein (data not shown).

Fig 4.3

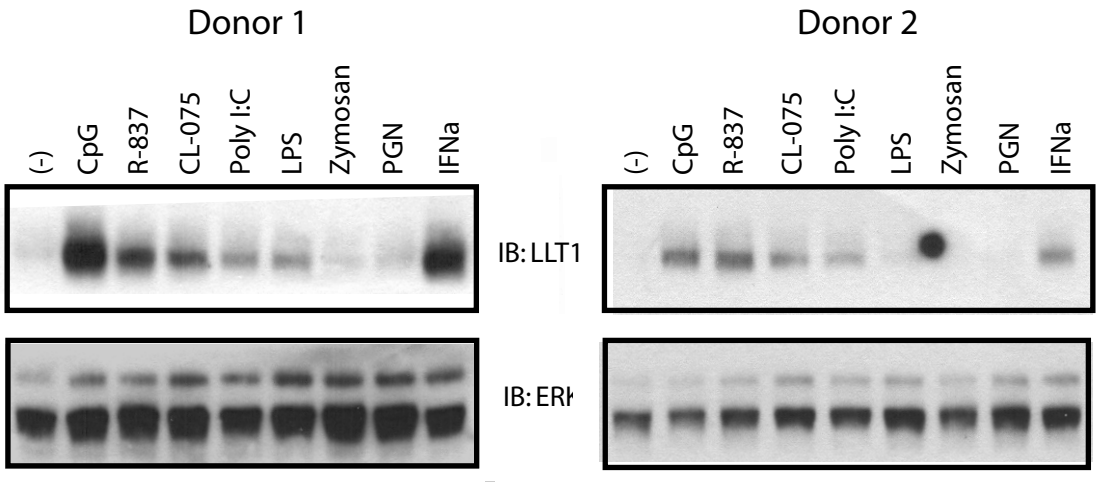

Figure 4.3. Toll-Like Receptor stimulations of peripheral blood low buoyant density mononuclear cells

A, Lysates of TLR stimulated low buoyant PBMC reveal that LLT1 is induced following treatment with TLR9 agonist CpG DNA, TLR 7 ligand R-837, TLR 8 ligand CL-075, TLR3 ligand poly I:C, TLR4 ligand LPS and IFNa, but not TLR2 lignads Zymosan and Peptidoglycan.

Activated plasmacytoid dendritic cells express LLT1

Because treatment with TLR9 ligand CpG DNA gave a potent response in B cells, we also examined LLT1 expression in plasmacytoid dendritic cells (pDCs) which also express TLR9 and are responsive to CpG DNA (109). pDCs, also known as interferon-producing cells (IPCs), respond to viral infections by rapidly producing high amounts of type I IFN. To determine if LLT1 is expressed by pDCs we examined microarray data from isolated pDCs treated with a panel of stimuli. We found that whereas freshly isolated pDC expressed little LLT1 mRNA, the amount of LLT1 was increased significantly after treatment with inactivated Flu virus, inactivated HSV, or CpG DNA of various compositions (Fig. 4.4a). To confirm these data, we performed quantitative RT-PCR on pDC cDNA and again saw upregulation of LLT1 transcripts following pDC activation with inactivated viruses or CpG DNA (Fig. 4.4b). Western blot analysis confirmed that LLT1 protein is induced in activated pDCs (Fig. 4.4c). LLT protein was induced strongest after stimulation with TLR9 ligand CpG-C DNA, TLR7/8 ligand R-848, and TLR7 ligand inactivated Flu virus.

We then examined whether DC other than pDC can express LLT1. We generated monocyte-derived dendritic cells (Mo-DC) by culturing $CD14^+$ sorted monocytes with IL-4 and GM-CSF for 6 days. Mo-DC were treated with various stimuli for 48 hours and assayed for LLT1 protein. Mo-DCs expressed LLT1 after stimulation with TLR3 ligand Poly I:C, TLR4 ligand LPS, and TLR 8 ligand CL-075 (Fig. 4.4d). Induction of LLT1 in MoDC is TLR specific as TLR2 ligands Zymosan & Peptidoglycan did not induce LLT1 protein.

Fig 4.4

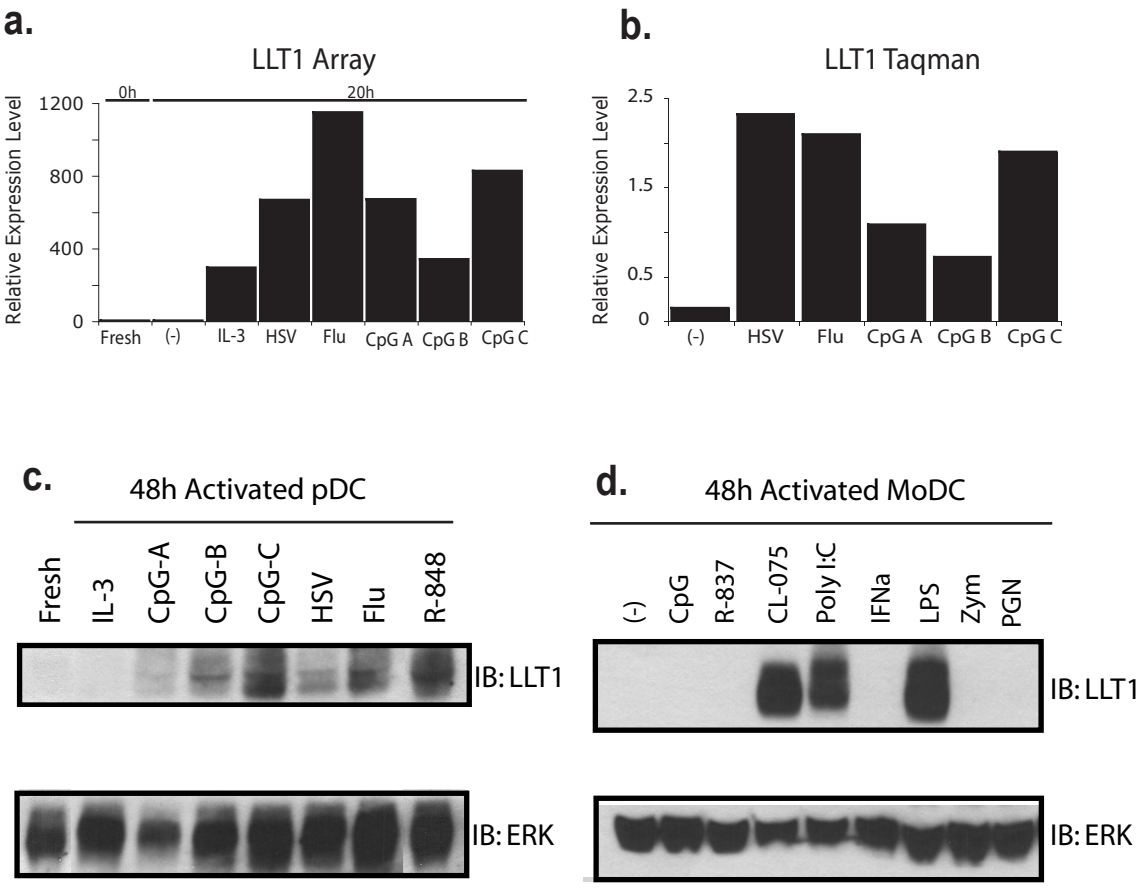

Figure 4.4 Activated plasmacytoid dendritic cells (pDC) express LLT1.

A, Microarray expression data from purified pDC samples demonstrate that pDCs treated with inactivated viruses or CpG DNAs induce LLT1 mRNA.

B, Quantitative RT-PCR of purified pDC samples confirm upregulation of LLT1 transcripts in activated pDCs.

C, Western Blots for LLT1 confirm that activated pDC samples express LLT1

D, Monocyte derived Dendritic cells (MoDC) activated with TLR3, TLR4, and TLR8 ligands express LLT1.

LLT1 on Raji cells does not affect TcR-dependent stimulation of NKR-P1A$^+$ CD4$^+$ T cells

In addition to being on NK cells, NKR-P1A is also expressed on CD4$^+$ and CD8$^+$ effector/memory T cell subsets (40, 101). NKR-P1A is expressed on approximately 30-50% of CD4$^+$ CD45RO$^+$ T cells and 30-60% of CD8$^+$ CD45RO$^+$T cells in adult human peripheral blood. Although an inhibitory role for NKR-P1A has been described for NK cells, no function has been previously reported for NKR-P1A on peripheral blood T cells. First, to confirm that LLT1 was a functional ligand on Raji cells, we co-cultured Raji cells with IL-2-activated peripheral blood NK cells and examined NK cell activation in the presence or absence of an anti-NKR-P1A F(ab)'$_2$ known to block the interaction with LLT1 (10). We examined NK cell degranulation by using CD107a (LAMP-1) surface staining, as well as IFNγ production by using intracellular cytokine staining. We observed a marked increase in both degranulation and cytokine production in the presence of blocking anti-NKR-P1A F(ab)'$_2$ (Fig. 4.5a). This demonstrated that Raji cells express functional LLT1, which serves to inhibit degranulation and cytokine production in NK cells through interactions with NKR-P1A.

Having validated that LLT1 on Raji cells functionally suppresses NK cell activation, we used these LLT1$^+$ Raji cells as antigen-presenting cells to stimulate NKR-P1A$^+$ T cells in the presence or absence of neutralizing anti-NKR-P1A F(ab)'$_2$. To enrich for resting effector/ memory T cells, we first isolated small, resting peripheral blood leukocytes from PBMC by Percoll density centrifugation, and then depleted cells positive for CD14, CD19, CD56, CD45RA, CD123, or CD236. T cells were cultured overnight, and then incubated with LLT1$^+$ Raji cells in the presence of superantigen Staphylococcus Enterotoxin B (SEB) at a suboptimal dose (based on prior titration) with or without

blocking anti-NKR-P1A F(ab)'$_2$. Cells were stimulated for 6 hours and analyzed for cytokine production by intracellular staining. We observed that although anti-NKR-P1A F(ab)'$_2$ enhanced NK cell activation, anti-NKR-P1A F(ab)'$_2$ did not affect SEB-induced $CD8^+$ T cell IFNγ or TNFα production (Fig 4.5b) or IL-2, IFNγ, or TNFα production by $CD4^+$ T cell (Fig. 4.5c). Experiments were performed at varying concentrations of SEB in the presence of saturating amounts of NKR-P1A F(ab)'$_2$, and no specific increase or decrease of cytokine production was noted (data not shown). Additional experiments performed in the presence of CTLA-4-IgG to block B7-CD28 interactions also showed no effect of blocking NKR-P1A (data not shown)

Fig 4.5

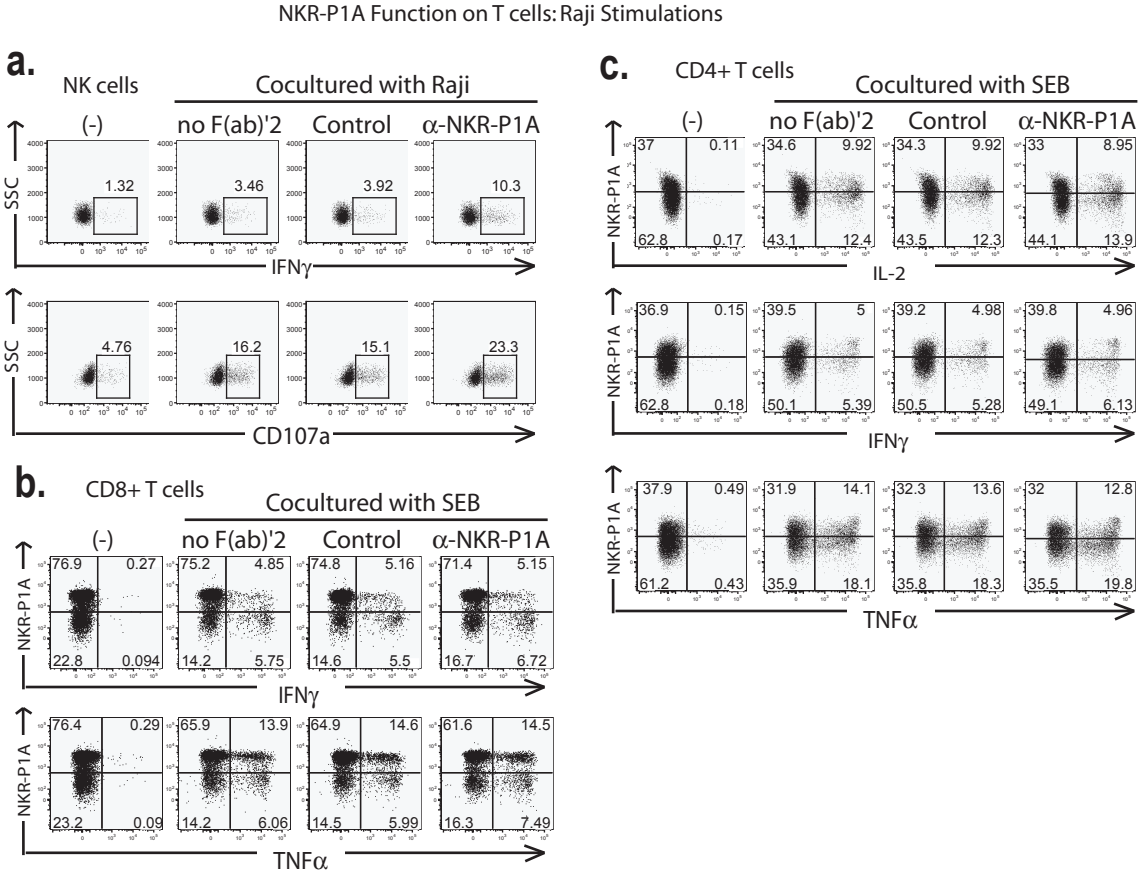

Figure 4.5 LLT1 on Raji cells inhibits peripheral blood NKR-P1A+ NK cells but does not affect peripheral blood NKR-P1A+ T cells.

A, Blocking NKR-P1A with F(ab)'2 reveals that LLT1 on Raji cells specifically interacts with NKR-P1A on primary human NK cells to functionally inhibit degranulation and cytokine production in NK cells.

B, Blocking NKR-P1A with F(ab)'2 reveals that LLT1 on Raji cells does not induce any specific effects on NKR-P1A+ CD8+ IFNg or TNFa production.

C, Blocking NKR-P1A with F(ab)'2 reveals that LLT1 on Raji cells does not induce any specific effects on NKR-P1A+ CD4+ IL-2, IFNg or TNFa production.

Anti-NKR-P1A mAb inhibits anti-CD3 mAb-induced TNFα production by CD8$^+$ T cells

To further assess the role of NKR-P1A in T cells versus NK cells, we used mAb-redirected activation with Fc receptor-bearing mouse P815 stimulator cells, a system we had previously used to study inhibitory receptors on NK cells (40, 112) and costimulatory receptors on T cells (108, 113). First, we demonstrated that anti-NKR-P1A mAb suppressed anti-CD244 (2B4) mAb-redirected degranulation of IL-2-activated peripheral blood NK cells. When the activating receptor CD244 (2B4) was cross-linked with anti-CD244 mAb by the Fc receptors on P815 target cells, we detected a significant increase in NK cell degranulation as measured by surface CD107a staining (Fig. 4.6a). However, when anti-NKR-P1A mAb, but not isotype-matched control antibodies, were added, we observed a marked decrease in NK degranulation, confirming an inhibitory role for NKR-P1A on NK cell function.

To test the role of NKR-P1A in T cells using this system, we co-cultured resting effector/memory T cells (isolated as described above) with P815 cells in the presence of sub-optimal concentrations of anti-CD3 and anti-CD28, with or without anti-NKR-P1A. We also used CD80 (B7.1)-transfected P815 cells and sub-optimal anti-CD3 mAb stimulation with similar results. We observed no specific differences in IFNγ or TNFα production by NKR-P1A$^+$ CD4$^+$ T cells when NKR-P1A was co-crosslinked with anti-NKR-P1A mAb by P815 cells (Fig. 4.6b). We did observe that a higher frequency of NKR-P1A$^+$ CD4$^+$ T cells produce IFNγ or TNFα than NKR-P1A$^-$ CD4$^+$ cells in this memory-enriched T cell population, in agreement with previously published findings (101). Because NKR-P1A inhibits NK cell degranulation (Fig. 4.6a), we tested the effects of NKR-P1A on T cell degranulation. We observed that whereas NKR-P1A$^+$

$CD8^+$ T cells degranulated after CD3 and CD28 stimulation, cross-linking NKR-P1A with mAb did not have any specific effects on degranulation in these cells (Fig. 4.6c). Lastly, we tested the effects of crosslinking NKR-P1A on cytokine production by NKR-$P1A^+$ $CD8^+$ T cells and found that whereas engaging NKR-P1A did not significantly effect IFNγ production, cross-linking NKR-P1A did result in a decrease in TNFα-producing cells in 3 of 7 blood donors analyzed (Fig. 4.6d).

Fig 4.6

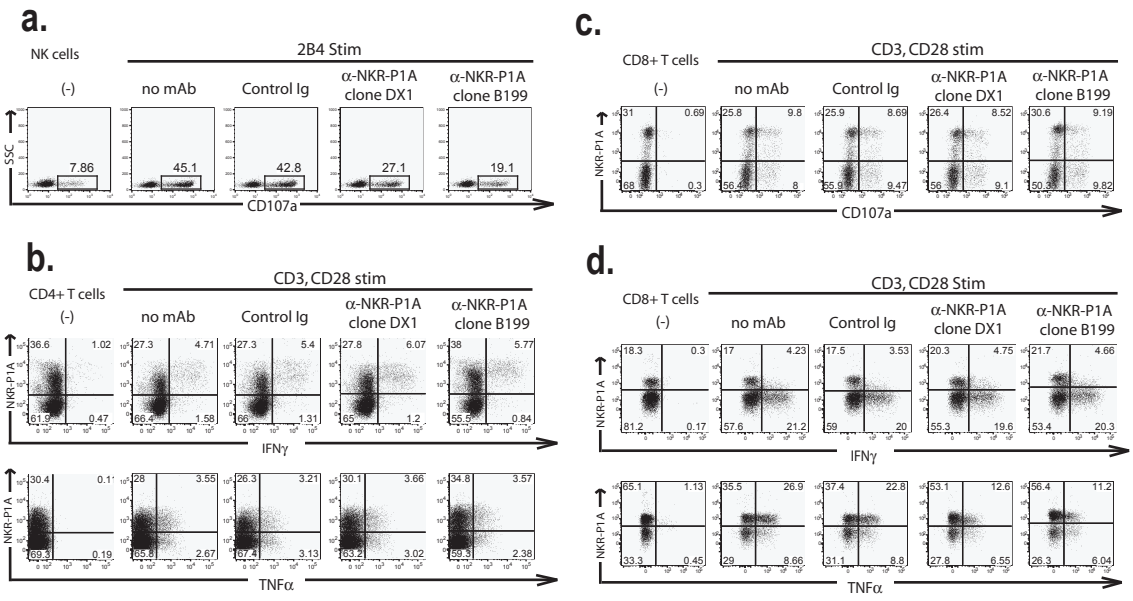

Figure 4.6 Crosslinking NKR-P1A with FcR+ P815 cells inhibits NK cell function and can inhibit NKR-P1A+ CD8+ T cell TNFα production.

A, Crosslinking NKR-P1A with P815 cells inhibits 2B4-induced degranulation in peripheral blood NK cells.
B, Crosslinking NKR-P1A does not change IFNγ or TNFα production by NKR-P1A+ CD4+ T cells.
C, Crosslinking NKR-P1A does not change degranulation on NKR-P1A+ CD8+ T cells.
D, Crosslinking NKR-P1A does not change IFNγ production by NKR-P1A+ CD8+ T cells but does inhibit TNFα production in 50% of donors analyzed.

Discussion

Although LLT1 was identified recently as a ligand for human NKR-P1A, little is known about the expression or regulation of this molecule or the physiologic relevance and context of this interaction. These experiments sought to define the context of this interaction by describing the cellular sources of LLT1 and by exploring the role of NKR-P1A on memory $CD4^+$ and CD8 T^+ cells, in addition to NK cells. We found that LLT1 was expressed on many B cell lines and on primary B cells activated by anti-CD40 or anti-IgM crosslinking or by treatment with TLR9 ligand CpG DNA treatment. we examined whether other TLR ligands could induce LLT1. Stimulation through TLR3, TLR4, TLR7, TLR8 & TLR9 induced LLT1 protein. As, TLR9 ligand CpG-B DNA always gave the strongest induction of LLT1 on B cells, we tested activated plasmacytoid dendritic cells might also express LLT1 as these cells have TLR9 and respond to CpG stimulation. TLR9 and TLR7 stimulated pDCs expressed LLT1 protein, as did TLR3, TLR4 or TLR8 stimulated monocyte-derived dendritic cells.

Additionally, we examined LLT1 expression on fresh monocytes, resting and IL-2 activated T cells, and resting and IL-2 activated NK cells and found no evidence for LLT1 protein by flow cytometry and Western blot analysis (data not shown), in contrast to prior reports (111). This was surprising as a previous report claimed that NK cells express LLT1 and that crosslinking LLT1 on NK cells induced the production of IFNγ (111). Using the L9.7 mAb (generously provided by Dr. P. Mathew), we failed to detect LLT1 on LLT1-transfected BaF/3 cells. It is also noteworthy that previous studies using this mAb reported a much higher molecular weight of LLT1 protein as analyzed by SDS-PAGE than we observed in our studies (111). We cannot explain the discrepancy

between our results and these previous reported findings, but are likely attributable to the nature of the mAbs used.

Prior studies from our lab and others have demonstrated that interactions between human NKR-P1A on NK cells and LLT1 on target cells inhibit NK cell-mediated cytotoxicity and cytokine production (10, 103). Our new findings that LLT1 is preferentially expressed on activated DC and B cells suggest that this molecule might regulate the interactions between NK cells and DC during viral infection or interactions between NK cells and activated B cells. pDCs can directly activate NK cells through mechanisms involving pDC-derived IFNα and GITRL (114, 115); however, activated pDC are known to be resistant to NK lysis.

As LLT1 is expressed on activated pDCs, it is possible that LLT1 upregulation on activated pDC might contribute to the resistance of these cells to NK cell-mediated lysis. Further studies will be required to explore the relative contribution of MHC class I and LLT1 on activated DC in protection against NK cell-mediated attack. Similarly, activated B cells expressing LLT1 might be protected, in part, from NK cell-mediated cytotoxicity via interaction with NKR-P1A. We have shown the potential for transformed B cells (e.g. 721.221 and Raji) to be protected from NK cells, and it is possible that normal B cells activated through their antigen-receptor or TLR9 might upregulate LLT1 to shield themselves from NK attack. Another consequence of LLT1 expression might be protection of tumors, particularly those low in MHC class I, from NK cell surveillance. In this regard, LLT1 was described recently as being expressed on malignant glioma cells (116). As LLT1 expression could represent a mechanism of immune evasion, it will be interesting to see if LLT1 is aberrantly expressed in cancer.

Although the interaction of LLT1 with NKR-P1A inhibits NK cell functions such as cytokine production and cytotoxicity, the functional consequences of LLT1 interacting with NKR-P1A on effector or memory T cells had not been addressed previously. We used LLT1$^+$ Raji cells and neutralizing mAbs against NKR-P1A to explore this question. Previous reports describing the function of NKR-P1A on human NKT cell clones and immature thymocytes using anti-NKR-P1A mAbs suggested a costimulatory role for this receptor on T cells (44, 45). In contrast, TcR-induced IFNγ or TNFα production by peripheral blood NKR-P1A$^+$ CD4$^+$ T cell was not affected by the presence of LLT1 on the APC (Raji B cells) or by cross-linking with an anti-NKR-P1A mAb that inhibited NK cell-mediated cytotoxicity and cytokine production. Similarly, NKR-P1A did not affect peripheral blood CD8$^+$ T cell degranulation. Lastly, we demonstrated that although NKR-P1A did not affect CD8$^+$ T cell IFNγ production, NKR-P1A did decrease anti-CD3-induced CD8$^+$ T cell TNFα production, suggesting a preferential inhibitory role on different effector functions. In contrast, NKR-P1A did not decrease SEB-induced CD8$^+$ T cell TNFα production, possibly because SEB and anti-CD3 stimulation differ or different cells were responsive to SEB versus anti-CD3. In comparing CD45RO$^+$ effector/memory T cell subsets, we found that, in agreement with previous reports, NKR-P1A$^+$ CD4$^+$ T cells produce more IFNγ and TNFα than their NKR-P1A$^-$ counterparts (101, 117). We also found that NKR-P1A$^+$ CD8$^+$ T cells are capable of degranulating and producing more TNFα than NKR-P1A$^-$ CD8$^+$ effector/memory T cells. Although NKR-P1A$^+$ CD8$^+$ T cells do produce IFNγ, they produce less than NKR-P1A$^-$ CD8$^+$ cells, in agreement with other studies (101)

Although previous experiments exploring the role of NKR-P1A on T cells have suggested a stimulatory role for this receptor, we have observed either an inhibitory role for this receptor on NK cell-mediated cytotoxicity or cytokine production or no effect of ligating this receptor on TcR-activated T cells in most assays. There are several possible explanations for the discrepancy in these various studies. First, most of the commonly used anti-NKR-P1A mAbs block the interaction between LLT1 and NKR-P1A (10, 103). Therefore, what appears to be "stimulation" might actually be caused by the anti-NKR-P1A mAb blocking the interaction between the inhibitory NKR-P1A receptor and its ligand on an antigen-presenting cell or target cell. In the studies demonstrating that anti-NKR-P1A mAb augmented the activation of immature thymocytes (45) and invariant NKT cell clones (44), it is possible that the anti-NKR-P1A mAb was actually blocking the interaction between an inhibitory NKR-P1A receptor on these T cells and LLT1 ligand being expressed by the NKT cell clones or thymocytes. In this regard, we have detected LLT1 protein by Western blot analysis in thymocyte lysates (data not shown). Alternatively, it is possible that the NKP-P1A on certain cell types, such as NKT cells or immature thymocytes, might possess activating function in certain situations. However, unlike in rodents, in humans there is only a single NKR-P1 gene and no evidence for alternatively spliced isoforms that can mediate distinct functional outcomes. Aside from inhibiting anti-CD3 mAb induced $CD8^+$ T cell TNFα production, we found no evidence that NKR-P1A enhanced or suppressed the TcR-induced activation of T cells, as determined by evaluating cytokine production and cytotoxicity. However, this does not rule out a role for NKR-P1A in other T cell functions. Other inhibitory C-type lectins, such as NKG2A, have been reported to inhibit TcR-induced apoptosis, thus it is possible

that NKR-P1A serves a similar role (118). We also examined anti-CD3-induced T cell proliferation and saw no role for NKR-P1A, although, interestingly, expression of NKR-P1A on the cell surface did go down after stimulation (data not shown)

NKR-P1A has the cytoplasmic sequence AIYAEL, which is similar to an ITIM (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val) - where X denotes any amino acid, except that NKR-P1A contains an alanine in the –2 position relative to the tyrosine residue (13). Previous data have suggested that this –2 position is important for inhibitory function and mutating the –2 position to an Alanine resulted in decreased inhibitory potential (119). This non-canonical ITIM likely explains the weak inhibitory potential of NKR-P1A. In mAb-redirected cytotoxicity experiments using the Fc receptor-bearing P815 target cell, crosslinking NKR-P1A inhibited anti-2B4-induced degranulation of human NK cells; however, crosslinking NKR-P1A did not significantly inhibit anti-CD16-induced degranulation of NK cells (data not shown), suggesting that the inhibitory potential of NKR-P1A can be masked by a strong activating stimulus. Thus, although NKR-P1A largely failed to modulate high-affinity anti-CD3 or SEB-induced responses, it remains possible that NKR-P1A does function in T cells physiologically to modulate low affinity TcR interactions, possibly against self-peptides. Collectively, our findings demonstrate that LLT1 can be induced on activated human B cells and DC and that the expression of LLT1 on B cell lines can inhibit NK cell-mediated cytotoxicity and cytokine production. Although under certain circumstances NKR-P1A might affect NKR-P1A-bearing T cell responses, it apparently does not suppress or augment activation induced by high affinity TcR engagement.

Chapter Five

Concluding Remarks

Natural Killer cells represent a third unique and specialized lineage of lymphocytes. Although T and B lymphocytes mainly use a single clonotypic antigen receptor to control their activity, NK cells process information from a variety of germline-encoded receptors to regulate their function. As NK cells do not require receptor editing or affinity maturation to be fully functional, NK cells are poised to rapidly respond to stressed or infected cells and so act as innate immune cells. As the effector functions of NK cells, such as cytotoxicity and cytokine production, can be enhanced by soluble factors such as IFNα and IL-12, NK cells are able to process information from neighboring immune cells. The activity of NK cells is also largely controlled by NK surface receptors, some of which activate and some of which inhibit NK cell function. In this fashion, NK cells can recognize and discriminate between healthy self, altered/stressed/infected self, and non-self and respond accordingly.

To investigate the mechanisms by which NK cells respond to stressed or infected cells, we examined the signaling pathways used by the activating receptor NKG2D in mice and humans. Whereas mouse NKG2D isoforms can functionally associate with both the transmembrane signaling adapter proteins DAP10 and DAP12, human NKG2D can only associate with DAP10. More specifically, the mouse NKG2D long isoform can only pair with DAP10, whereas the mouse NKG2D short isoform can pair with either DAP10 or DAP12 In contrast, human NKG2D or an artificially truncated "short" human NKG2D can only pair with DAP10. We found that the species-specificity of adapter protein usage is determined by the transmembrane domains of NKG2D. Evidence from other groups supports our conclusion that signal adapter specificity is determined by

transmembrane interactions (120). Recently, the stoichiometry of human NKG2D and DAP10 was described as a hexameric complex, comprising one NKG2D homodimer and two DAP10 homodimers (121). An interesting experiment would be to see if one mouse NKG2D homodimer is capable of associating with one DAP10 homodimer and one DAP12 homodimer simultaneously, which could readily be addressed by co-immunoprecipitation experiments. As DAP12 has ITAMs and more potently induces cytokine production than DAP10, mouse NKG2D signaling seems to be more potent than human NKG2D. However, interestingly, DAP12 has recently been shown to also have an inhibitory role in immune cells under some circumstances (122, 123). It is possible that the number and type of DAP12-associated receptors will impact that cell's signaling activity. For example, if a cell expresses too many DAP12-associated receptors then DAP12 may become rate-limiting and diminish signaling through these receptors. Perhaps the difference between human and mouse NKG2D's use of DAP12 relates more to the total amount of DAP12-associated receptors in the cell rather than to the specific signaling potential of NKG2D. In other words, human NK cells might have more DAP12-associated receptors and therefore may have evolved not to use DAP12 with NKG2D to keep the cell's potential activity higher. In any event, the differences we see in mouse and human NKG2D illustrate how this receptor signals more potently in mouse cells and demonstrate differences between the mouse and human immune system. It is vital for scientists to keep these differences in mind because the mouse is a widely used pre-clinical model system for human therapeutics. In that regard, it would be interesting to construct an NKG2D knock-in mouse containing the human transmembrane domain.

This would more closely mimic the human biology and would serve as a closer animal model for human NKG2D biology.

In our studies, we were unable to specifically determine which portion of human and mouse NKG2D transmembrane sequences defined DAP10 and DAP12 specificity. Among the mutations examined, we swapped the human FCCF sequence for the mouse VVRV sequence, and examined the unique mouse FQPV sequence and found that changing these sequences did not affect adaptor usage. We also found that a Macaque NKG2D transmembrane-CD69 Extracellular chimeric protein bound DAP10 but not DAP12, like human NKG2D-CD69 chimeras. Studies examining charged transmembrane interactions would suggest that the position of the charged residue mainly determines permissibility for adapter usage (124), however as mouse and human NKG2D transmembrane sequences have their charged Arg residues in the same position, other transmembrane sequences must be playing a role, either stabilizing DAP12 in mouse NKG2D or inhibiting a DAP12 interaction with human NKG2D. A comparison of other species NKG2D transmembrane sequences, swapping residues between mouse and human sequences, or an alanine scanning approach would provide insight into the required residues. Interestingly, in the transmembrane adapter protein CD3ζ, a single amino acid difference between mouse (Ile-46) and human (Leu-46) CD3ζ, allows human CD16 to pair well with human, but not mouse, CD3ζ {Κυροσακι, 1991 #1506}. This finding highlights how difficult it can be to predict transmembrane interactions based on sequence alone and suggests the need for more experimentation to clearly define the requirements for transmembrane interactions between DAP10 or DAP12 dimers and NKG2D.

To further understand how the activity of human NK cells is regulated, we explored the biology of cell surface receptor, NKR-P1A. Like NKG2D, NKR-P1A is a type II transmembrane disulphide-linked homodimer. However, unlike NKG2D, which is an activating receptor, previous reports of NKR-P1A function suggested an inhibitory role. After reports surfaced regarding the mouse NKR-P1 family members interacting with clr ligands (48, 49), we examined whether human NKR-P1A might recognize the human clr homolog, LLT1. Using binding studies as well as reporter cells, we found that LLT1 is a ligand for human NKR-P1A. NK cells exhibited reduced cytotoxicity when LLT1 was expressed on target cells, and this inhibition could be reverted by addition of blocking anti-NKR-P1A F(ab)'$_2$, demonstrating that LLT1 interacts with NKR-P1A to inhibit NK cell-mediated cytotoxicity. Interestingly, the genes encoding LLT1 and NKR-P1A lie adjacent to each other in the genome and the two most related proteins, AICL and NKp80, respectively, were recently shown to interact, providing another example of genomically linked C-type lectins functioning in receptor-ligand relationships (105).

To further explore the physiologic relevance of this NKR-P1A-LLT1 interaction, we examined the distribution of LLT1 in the human immune system. We found that many B cell lines express LLT1 and primary B cells express LLT1 when activated, most potently by CpG DNA treatment. In examining other TLRs, we found LLT1 induced after stimulation through TLR3, TLR4, TLR7, TLR8 & TLR9. We saw induction of LLT1 in TLR7 and TLR9 stimulated pDCs and TLR3, TLR4 or TLR8 stimulated monocyte-derived DCs. This is likely representative of primary myeloid DC as primary myeloid DCs do upregulate LLT1 transcript after treatment with TLR3 ligand poly IC and TLR7/8 ligand R848 (W. Cao, unpublished array data). Previously, LLT1 had been

described as an “NK receptor” based on transcription in certain NK cell lines; however, no reliable reports of LLT1 protein on primary NK cells have been described. We looked for LLT1 on NK cells and did not find any LLT1 protein on resting or activated normal peripheral blood NK cells, with the exception that PMA and ionomycin did induce barely detectable amounts of LLT1 on NK cells. However, we did find LLT1 protein on the transformed NK cell lines NKL and YT2C2 and detected increased amounts of LLT1 after PMA and ionomycin treatment of these cell lines. In contrast, we found LLT1 on activated B cells, pDC, and monocyte-derived DCs, which may protect these cells from NK cell- mediated cytotoxicity. Indeed, experiments with autologous B cells activated with CpG and IgM to express LLT1 demonstrated that LLT1 expression on primary B cells inhibits autologous NK cytotoxicity (Fig 5.1). As NK cells and pDCs are capable of cross-activating each other (115, 125), this NKR-P1A-LLT1 interaction likely ensures maximal activation of both cell types, without harm to the pDC.

Fig 5.1

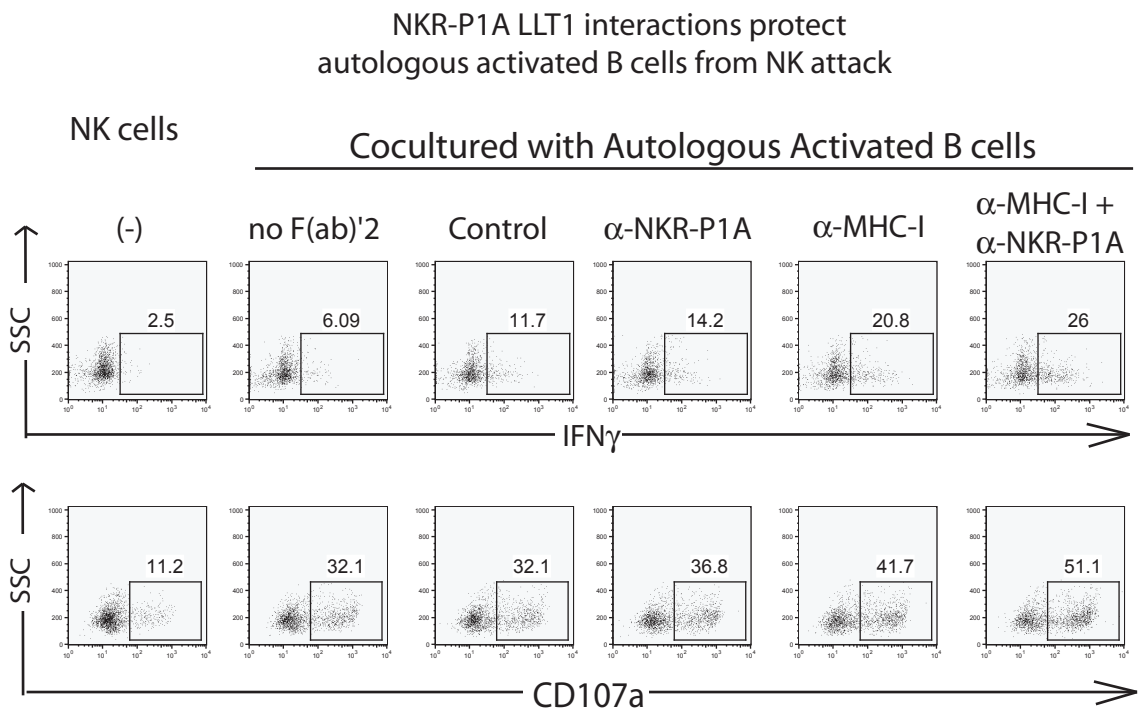

Figure 5.1 LLT1 on activated B cells inhibits autologous NK activation.

CpG, IgM stimulated B cells were cultured with autologous IL-2 activated NK cells in the presence or absence of blocking F(ab)'2s against NKR-P1A. More Degranulation and IFNg production by NK cells is seen when NKR-P1A - LLT1 interactions are blocked.

As many B cell lines and activated B cells express LLT1, we also examined if any B cell cancers might aberrantly express this molecule. We found that in both multiple myeloma and B cell-derived ALL samples, LLT1 did not seem to be overexpressed. We also examined Chronic Lymphocytic Leukemia, CLL, and detected increased amounts of LLT1 mRNA in ~30% of samples analyzed (data not shown). Unfortunately we were not able to obtain any viable CLL cells for functional analysis. As such, examining LLT1 protein expression and function in CLL samples remains as an exciting future direction for this project. Examining other cancer types for elevated LLT1 levels should be an exciting and informative future line of experimentation because LLT1 expression by CLL or other cancers could function as a mechanism of immune evasion to avoid NK cell surveillance.

30-50% of $CD45RO^+$ $CD4^+$ or $CD8^+$ T cells express NKR-P1A. To address the role of NKR-P1A on T cells we first used $LLT1^+$ Raji cells and superantigen SEB to stimulate T cells. Whereas NKR-P1A on NK cells recognized LLT1 on Raji cells and inhibited NK cell-mediated cytotoxicity and IFNγ production, engaging NKR-P1A on $CD4^+$ or $CD8^+$ T cells neither increased nor decreased SEB-induced cytokine production (IL-2, IFNγ, and TNFα). As Raji cells express B7, ICAMs, and other costimulatory molecules, we then took a more reductionist approach using antibody-redirected stimulation with the Fc-receptor-bearing P815 cells. Using this system, we found that NKR-P1A on NK cells could inhibit degranulation induced by anti-2B4 antibody, which stimulates the activating 2B4 receptor on human NK cells. Moreover, we found that NKR-P1A on $CD8^+$ T cells could inhibit TNFα production in three out of seven donors

analyzed. In contrast, we saw no specific effect on cytokine production by cross-linking NKR-P1A on TcR-activated $CD4^+$ T cell cells and no impairment of anti-CD3-induced degranulation by $CD8^+$ T cells. As NKR-P1A has a "non-canonical" ITIM with presumably weaker inhibitory capacity, it still remains possible that NKR-P1A plays a role with lower affinity TcR interactions. Alternatively, there exists the possibility that NKR-P1A really has no function in T cells and its expression is merely reflects the differentiation state of these cells. One interesting experiment would be to mutate the –2 Alanine to a typical ITIM residue such as Ile/Val/Leu/Ser and test if NKR-P1A with a canonical ITIM is more capable of inhibiting NK cell and T cell responses.

Interestingly, higher proportions of NKR-$P1A^+$ T cells have been reported in the intestine (126, 127). This is not surprising as many NKR-P1A expressing $CD4^+$ and $CD8^+$ T cells express the intestine-homing receptor $\alpha4\beta7$ Integrin ((128) and data not shown). It is tempting to speculate on what NKR-P1A might be doing in the gut and/or if intestinal cells express LLT1. In addition, data exist that the frequency of NKR-$P1A^+$ T cells is increased in cancer patients (117), which raises the question of what role, if any, NKR-P1A may be playing in cancer.

Overall, the NK genomic complex (NKC) represents a region rich in locus encoding interesting immune cell receptors, including NKG2D, NKR-P1A, and LLT1. Whereas mice have multiple *Nkrp1* and multiple *Clr* genes, humans only have one ortholog of each, *KLRB1 (*NKR-P1A) and *CLEC2D* (LLT1). In fact, among species, humans, dogs, and cattle all have only one *KLRB1* and one *CLEC2D* gene, whereas mice and rats have multiple *KLRB1* and multiple *CLEC2D* genes (129). Interestingly, mice and rats uniquely have an expanded NKC overall with 57 and 75 genes, respectively,

compared to 29 genes in humans, 22 in dog, and 32 genes in cattle (129). It will be interesting to explore the functions of the various *KLRB1* (*Nkrp1) - CLEC2D (Clr)* interactions in mice and to see how the expansion of these loci provide different immune functions. A few simple experiments could also determine if the *KLRB1* and *CLEC2D*-encoded proteins also function as receptors and ligands in other species, such as cattle, dogs, and chimpanzees. If they do interact, examining the expression pattern of these NKR-P1A and LLT1 orthologs in other organisms will illuminate how conserved this interaction is among species.

Thus, to more completely understand how NK cells are regulated by surface receptors, we have examined the mechanisms by which NK cells signal through the NKG2D receptor and have defined the LLT1-NKR-P1A relationship as a non-MHC class I inhibitory interaction for regulating human NK cell activity. This suggests another way for activated lymphocytes to protect themselves from NK editing and attack and asks new questions about the role NK cells play in regulating immune homeostasis.

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