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A Thesis for the Degree of Doctor of Philosophy

Signaling Mechanisms Modulating
Synergistic Activation of Natural Killer Cells

자연살해세포의 시너지 활성을 조절하는
신호전달 기전

August, 2016

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The Interdisciplinary Program in Agricultural Biotechnology

College of Agriculture and Life Sciences

Seoul National University

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이 논문을 박사학위논문으로 제출함
2016년 8월

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Signaling Mechanisms Modulating Synergistic Activation of Natural Killer Cells

Abstract

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Natural Killer (NK) cells are discriminated among innate lymphoid cells (ILCs) with their cytolytic activity that has a key role in the control of viral infection and tumorigenesis. NK cells not only kill virus-infected or transformed cells including cancer stem cells by releasing cytotoxic granules, but also contribute to modulate diverse immune responses by secretion of cytokines (e.g., IFN- γ and TNF- α) and chemokines (e.g., MIP-1 α and MIP-1 β). Therefore, the malfunction of NK cells is linked various diseases, and the manipulation of NK cells function have hold promise in therapeutic applications. However, the limited information for the mechanisms regulating activation of NK cells has hampered the progression toward therapeutic strategies. Accordingly, understanding accurate activating and inhibitory receptors signaling mechanisms controlling NK cells function can augment successful NK cell-based therapies.

The triggering of NK cell effector functions relies on the engagement of activating or inhibitory receptors for ligands on their target cells. The balanced

signals from activating and inhibitory receptors are known to dictate NK cells' activity. However, even in the case of inhibitory signal is absent, activating signal from single coactivation receptor is insufficient to induce enough activation of resting NK cells. Signals from multiple activating receptors, not a single receptor, are required for NK cell functions. And only specific combinations among them can induce additive or synergistic enhancement of NK cells' activity. For this reason, the study of signaling mechanisms modulating synergistic activation of NK cells is critical for harnessing the 'arm' of NK cells for clinical purposes. In this study, two critical signaling molecules for immune cell functions, glycogen synthase kinase (GSK)-3 β and NF- κ B are examined for regulation of synergistic activation of NK cells, and their interaction mechanisms with other signaling pathways are investigated.

In a synergistic activation model of NK cells that combines NKG2D and 2B4 activating receptors, representative non-ITAM-associated activating receptors, GSK-3 β negatively regulated the synergistic activation of both cytotoxicity and cytokine secretion. The individual or combinational stimulation of NKG2D and 2B4 induced inhibitory phosphorylation of GSK-3, and the extent of phosphorylation in accordance with the level of NK cell activity. The inhibition of GSK-3 β by siRNA knockdown or pharmacological inhibition increased NK cell function, but GSK-3 α knockdown had no effect. This negative role of GSK-3 β was dependent on its kinase activity. The regulation of NK cell function by GSK-3 β was a common mechanism for both NKL cell line and primary NK cells. And NK cell activations by either ITAM-associated or non-ITAM activating receptors were regulated by GSK-3 β .

NF- κ B is a well-known transcription factor that is critical for diverse

immune responses. Nevertheless, its role in NK cells activated by multiple activating receptors during target cell recognition is not defined. Synergistic combination of NKG2D, 2B4, or DNAM-1 induced synergistic and sufficient activation of NF- κ B pathway that is linked to synergistic NK cell activity, whereas single receptor stimulation was insufficient. Receptors cooperation for NF- κ B activation was regulated at the level of Vav1 phosphorylation, the 1st checkpoint, and this Vav1-dependent synergistic signal also cooperates with separate PI3K-Akt signal for synergistic p65 phosphorylation, the 2nd checkpoint, in a stepwise manner. As confirmed with a X-linked lymphoproliferative disease (XLP) NK cells model that is defective for 2B4 receptor signaling because of mutations of SAP adaptor, stepwise checkpoint signaling mechanism for NF- κ B activation was suggested.

These newly uncovered signaling mechanisms during multiple receptor stimulation provides new insights of receptor cooperation to determine specificity and magnitude of NK cell activation and bases for establishing NK cell-based therapeutic strategies.

Key words: Natural Killer cells, Synergistic activation, Receptor signaling, GSK-3 β , NF- κ B, XLP

Student Number: 2008-30923

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List of Abbreviations

ADCC – antibody-dependent cell-mediated cytotoxicity

AML – acute myeloid leukemia

CA – constitutively active

DC – dendritic cell

DNAM-1 – DNAX accessory molecule-1

EBV – Epstein-Barr virus

ELISA – enzyme-linked immunosorbent assay

ERK – extracellular signal-regulated kinase

FACS – Fluorescence-activated cell sorting

GSK-3 – glycogen synthase kinase-3

GVHD – graft-versus-host disease

HLA – human leukocyte antigen

IFN - interferon

IKK – I κ B kinase

ITAM – immunoreceptor tyrosine-based activation motif

ITIM - immunoreceptor tyrosine-based inhibition motif

ITSM – immunoreceptor tyrosine-based switch motif

KD – kinase dead

KIR – killer-cell immunoglobulin-like receptor

LAT – linker for activation of T cell

MAPK – mitogen-activated protein kinase

MCMV – mouse cytomegalovirus

MEK – MAPK/ERK kinase

MHC – major histocompatibility complex

MIC – MHC class I polypeptide-related sequence

MIP – macrophage inflammatory protein

NCR – natural cytotoxicity triggering receptor
NFAT – nuclear factor of activated T cell
NF- κ B – nuclear factor κ B
NK – natural killer
NTAL – non-T-cell activation linker
PBMC – peripheral blood mononuclear cell
PI3K – phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC – protein kinase C
PLC – phospholipase C
PLGF – placental growth factor
SAP – SLAM-associated protein
SHP – Src homology region 2 domain-containing phosphatase
siRNA – small interfering RNA
SLAM – signaling lymphocytic activation molecule
SLP-76 – SH2 domain-containing leukocyte phosphoprotein of 76 kD
TDZD - thiadiazolidinone
TNF – tumor necrosis factor
TRE – transcription response element
ULBP – UL16 binding protein
VEGF – vascular endothelial growth factor
WT – wild type
XLP – X-linked lymphoproliferative disease
YINM – tyrosine-based signaling motif

Chapter I.

General Introduction

I-1. Research background

I-1-1. Cancer therapies

It is well-recognized that cancer is an old-age disease because cancer incidence increases with an extended life expectancy caused by progress of health care technology. Most primary tumors are now being treated by combinational therapy including surgery, chemotherapy, and radiation. But these conventional therapies can cause immunosuppressive side effect and tumor relapse by micrometastasis of residual cancer stem cells (1, 2). For this reason, targeted cancer therapy, interfering specific target molecules required for tumor cell cycle, is expected to be more safe and effective than conventional therapies. However, targeted therapy also has limitation that most cancer cells' phenotype is resulted from multiple genes' modification while current targeted therapies are focusing on a single target (3). And it is effective only before tumor is resistant to targeted therapeutic agent. Moreover, tumor progression is not only dependent on cell-autonomous gene alterations, but also dependent on microenvironment including cytokines and immune responses against tumor cells (4). The strategy exploiting the host's immune system for cancer treatment depends on the insight that immune system can surveil initial transformation and eradicate residual cancer stem cells after therapies (5). Cancer immunotherapy, ranging from passive to active, encompasses cancer vaccines, adoptive cellular immunotherapy, immune checkpoint blockade, and

oncolytic viruses (6). Increasing clinical studies have combined these conventional therapy and immunotherapy and suggest that combinations can create a synergy (7).

I-1-2. NK cell-based cancer therapy

Allogeneic hematopoietic stem cell transplantation (HSCT) is an established strategy for cancer immunotherapy, especially for hematological malignancies (8). The patients are treated by irradiation and/or chemotherapy prior to transplantation to suppress the recipient's immune rejection and are through the introduction of competent donor immune cells. This donor immune system can trigger graft-versus-tumor (GVT) effect. The patients with acute myeloid leukemia (AML) undergoing HSCT, in which a KIR-ligand mismatch exists, showed improved survival rate and reduced relapse rate (9, 10). After transplantation, donor T cells may become activated by recipient antigen-presenting cells (APC) and kill residual tumor cells. In the meantime, these T cells may also cause tissue damage referred to as 'graft-versus-host disease' (GVHD). NK cells are also known to mediate GVT effect, but not to involve GVHD. They rather decrease GVHD inducing inefficient priming of donor T cells by killing recipient's APCs (11). Several strategies were suggested to take advantage of this strong point of NK cells, such as depleting T or B cells and *ex vivo* expansion of NK cells (12, 13). In addition, several strategies enhancing transferring NK cells' activity, by means of cytokines or modulation of activating or inhibitory receptors, are suggested. But the establishment of NK cell-based therapy remained difficult because of the lack of

information about mechanisms for NK cell activation and requires for detailed investigations.

I-2. Innate lymphoid cells

I-2-1. Classification of innate lymphoid cells

NK cells belong to innate lymphoid cells (ILCs), a recently expanding family of innate immunity (14). Unlike T or B cells, ILCs are a group of lymphocytes that do not have rearranged antigen-specific receptors (15). ILCs are classified on the basis of transcriptional and functional profile. Similar to T cells classification, ILCs are also grouped into cytotoxic- and helper-ILC, and NK cells belong to cytotoxic-ILCs (16). Helper-ILCs further divided into ILC1, ILC2, and ILC3. ILC1 development depends on T-bet transcription factor and secrete IFN- γ . ILC2 depends on GATA3 transcription factor and secrete type 2 cytokines, such as IL-5 and IL-13 (17). ILC3 express ROR γ t and secrete mainly IL-17 and IL-22 (18). But this information for ILC development is mostly from mice studies, and less is characterized in humans (19). In the meantime, a recent study found common lymphoid progenitor population in secondary lymphoid tissues that express ROR γ t and generate all known ILC subsets, implying different system in humans (20). With diverse cytokine profile and function, ILC subsets contribute to defenses against a variety of pathogens (21, 22). Their role for host defense is crucial in immune-compromised mice (23), while it seems to be marginal in the presence of a functional T cells. In humans, the role of ILCs including NK cells may become more important in case of T cell-depleted HSCT recipient to prevent opportunistic infections.

Therefore, establishment of strategies for NK cell-based cancer therapy has to consider the role of whole ILC population including both cytotoxic- and helper-ILCs.

I-2-2. Differences of NK cells from other ILCs

Conventional NK cells were initially classified in group 1 ILCs as they express transcription factor T-bet and secrete IFN- γ . However, NK cells are developmentally dependent on Eomesodermin (Eomes), not on T-bet. In addition, NK cells express cytotoxic molecules, such as perforin and granzyme, that mediate their cytotoxic activity. This cytotoxic function of NK cells discriminates themselves from other ILCs as an innate counterpart of CD8⁺ cytotoxic T cells (24). NK cells are also different from other ILCs for the independency on IL-7. NK cells are IL-7R α negative while other helper-ILCs are IL-7R α positive.

NK cells hold their specialized position as critical innate effectors for anti-tumor and anti-viral immunity in spite of characterization and expansion of ILC family.

I-3. Functional characteristics of NK cells

I-3-1. NK cells

NK cells had been named as large granular lymphocytes with ‘natural’ cytotoxic activity to target cells without priming or inhibition. And now they are categorized as separate lymphoid lineage with both cytotoxicity and cytokine secretion activity (25-28). Following studies have proposed that NK cells recognize and kill target cells that are lack of self MHC class I molecules (“Missing self” hypothesis) (29, 30) and then found inhibitory receptors discriminating non-self from self cells. Thereafter, discovery of diverse activating NK cell receptors changed the activation of NK cells against ‘missing self’ target cells from nonspecific response to highly organized process with ‘dynamic balance’ (31). Although NK cells are lack of dominant antigen receptors, expressed by T or B cells, they express various array of germ-line encoded receptors. And their multiple engagement during target cell recognition result in the outcome of NK cell activity (32, 33). Activating receptors of NK cells sense ligands on stressed, transformed, or virus-infected cells, such as ULBP and MIC molecules for NKG2D receptor (34). Activation of NK cells depends not only on ligands of target cells, but also on cytokine microenvironment and interaction with other immune cells (35). NK cells can be activated by type I IFN, IL-12, IL-18, and IL-15 (36), and CD4⁺ T cell-derived IL-2 enhances NK cell functions (37). NK cells also can be regulated by transforming growth factor (TGF)- β produced by regulatory T cells (38, 39). Cytokines, such as IFN- γ or TNF- α , secreted by NK cells also shape other immune cells responses (40, 41).

NK cells, with these sophisticated regulation mechanisms, interpret their environment and perform specialized tasks in anti-tumor or anti-viral immunity and immune cells cross-talk.

I-3-2. Anti-tumor activity of NK cells

Many *in vivo* and *in vitro* studies advocates the anti-tumor activity of NK cells (25). NK cells in mouse show *in vivo* rejection of several transplanted tumors in a NK receptor-target cell ligand-dependent manner (42). In some experiments, anti-tumor activity of NK cells induces generation of specific T cell responses against the same tumor cells (43) that could be a strong point of NK cell-based therapeutic strategy. In addition, immunosurveillance for spontaneous or induced tumors by NK cells was reported (44). In humans, although the characterization of NK cell function *in vivo* is difficult because of the lack of NK cell-specific deficiency (45), stacked epidemiologic survey suggests that the level of NK cell activity in peripheral blood is correlated with cancer risk (46). And allogeneic hematopoietic stem cell transplantation into recipient tumor patient, such as acute myeloid leukemia (AML), lacking donor KIR ligands leads to the development of donor NK cells and better post-transplant survival without graft-versus-host disease (GVHD) (47). In other mouse *in vivo* experiment, the blocking of NK cell inhibitory receptor specific for MHC class I molecules increased anti-tumor activity of NK cells (48) and this approach is in clinical trial in human AML. Understanding molecular mechanisms for NK cell activation is expected to strengthen this potential of NK cells as a tool for cell-based anti-tumor therapy.

I-3-3. Anti-viral activity of NK cells

Important clues about anti-viral activity of NK cells were obtained by NK cell depletion or adoptive transfer studies with mice. Deficiency of NK cell activities including cytotoxicity or cytokine production increased the susceptibility of mice to the herpesvirus MCMV infection (49, 50). NK cells recognize cell surface ligands, such as m157, encoded by CMV, on MCMV-infected cells with NK cell activating receptor Ly49H (51, 52). The struggling of MCMV with genes that play a role in evasion of NK cell recognition also emphasizes the critical role of NK cells in virus infections. Among these genes are involved in downregulation of ligands for NKG2D or in expression of MHC class I homologs that stimulate inhibitory receptors on NK cells (53).

Another emerging aspect in anti-viral activity of NK cells is regulation of immunopathology (54). NK cell depletion in mice leads to accelerated the encephalitis by Theiler's virus or myocarditis by coxsackie B3 virus (55, 56). NK cells promote the trigger of CD8⁺ T cell responses and so control type I IFN-induced immunopathology by virus dissemination (57). NK cells also suppress over-activated macrophages with their cytotoxic activity (58).

Therefore, NK cells perform dual mechanism during virus infection by eliminating both virus-infected cells and over-activated pathologic immune cells.

I-3-4. Crosstalk of NK cells with other immune cells

NK cells interact with other immune cells, such as DCs, T cells, and B cells,

by means of both cytotoxicity and cytokine secretion. NK cells interact and influence DCs in two different ways (59, 60). NK cells can maintain DC homeostasis by eliminating immature DCs (61). On the other hand, NK cells promote DC maturation by secretion of IFN- γ and TNF, and also are activated by IL-12 secreted by DCs (62). In the interaction with T cells, NK cells can help the priming of type I helper T cells by secretion of IFN- γ (63). NK cells also kill activated T cells that are without enough MHC class I molecules (64). Therefore, blocking inhibitory receptors can enhance the cytotoxic activity of NK cells against CD4⁺ T cells, that is applicable to the therapy for T cell-mediated autoimmunity. NK cells are also known to suppress autoreactive B cells (65). Distinct subset of NK cells in uterine can interact with endothelial cells by promoting angiogenesis in case of pregnancy (66). These NK cells secrete vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) (67). Accordingly, effector functions of NK cells are not limited to anti-cancer or anti-viral activity, but also to broad immune-modulatory activity.

I-4. Inhibitory and activating receptors of NK cells

I-4-1. Inhibitory receptors and signaling

The discovery of immunoreceptor tyrosine-based inhibition motif (ITIM) as a site with a sequence motif for binding of tyrosine phosphatase SHP-1 to inhibitory killer cell Ig-like receptors (KIR) in human (68) triggered the finding of many other ITIM-associated receptors (69). The KIR family in human binds classical MHC class I molecules, such as HLA-B and HLA-C. The Ly49 family in mice binds H-2 class I molecules. The CD94-NKG2A, that is conserved in human and mice, binds nonclassical MHC molecule HLA-E in human and Qa1 in mice. In spite of the structural diversity between KIR and Ly49 and CD94-NKG2A, their signaling through ITIM is remarkably conserved.

Signaling by inhibitory receptors starts with phosphorylation of their ITIM motif. Phosphorylated ITIM recruits the tyrosine phosphatases SHP-1 and SHP-2. KIR is also known to bind additional molecule, β -arrestin 2 (70). The lack of β -arrestin 2 downregulates the inhibition by KIR because β -arrestin 2 facilitates SHP-1 and SHP-2 recruitment to KIR. The inhibition by ITIM-bound SHP is at very proximal signaling step, which is through dephosphorylation of Vav1 by SHP-1. Given the critical role of Vav1 for actin cytoskeleton rearrangement, synapse formation, and receptors clustering, this proximal inhibitory process suppresses the tyrosine phosphorylation of activating receptors even before it begins (71, 72). The inhibitory signaling after binding to MHC class I ligand also can be through tyrosine phosphorylation of small adaptor Crk that forms a complex with Abl, a process

dissociating Crk from scaffold protein Cbl (73).

Inhibitory signaling also play a role for NK cell education process, which is determined by MHC class I repertoire (74). NK cells that have detected more MHC molecules by their inhibitory receptors become more responsive. Conversely, NK cells with no contact for MHC become hyporesponsive. Two education mechanisms are suggested, that is anergy induced by continuous stimulation without enough inhibition (75) or acquisition of responsiveness by instructive signal through inhibitory receptors (76).

I-4-2. Activating receptors and signaling

Instead of a dominant antigen-specific receptor for activation, such as TCR or BCR, NK cells express diverse receptors which can activate effector functions in synergy (77). These activating receptors recognize ligand expressed on virus-infected, stressed, or transformed cells (78, 79). The resting human NK cells can be activated only by combination of activating receptors, which is called coactivation receptors, not by single receptor stimulation (80). Whereas, an exceptional receptor is the low-affinity Fc receptor Fc γ RIIIa (CD16), which recognize Fc region of antibody and is sufficient alone to activate resting NK cells for antibody-dependent cellular cytotoxicity (ADCC). In contrast to inhibitory receptors, which has varied expression in distinct peripheral blood NK cell subsets, activating receptors are expressed mostly by all NK cells. Moreover, activating receptors induce diverse signaling pathways, while inhibitory receptors use common mechanism for inhibition.

Activating receptors containing immunoreceptor tyrosine-based activation

motif (ITAM) or associated with adaptors carrying ITAM includes CD16, natural cytotoxicity receptors (NCRs), such as NKp30, NKp44, and NKp46, and activating KIRs, such as KIR2DS and KIR3DS, and CD94/NKG2C heterodimer (77). These receptors, upon ligand engagement, are tyrosine-phosphorylated by Src family kinases and recruit tyrosine kinases, such as Syk and ZAP-70. Syk and ZAP-70 kinases phosphorylate linker for activation of T cells (LAT) and non-T-cell activation linker (NTAL) followed by activation of PI3K, PLC- γ 1, 2, and Vav2, 3. (81, 82).

Among non-ITAM receptors are NKG2D, CD2, 2B4, CRACC, NTB-A, and DNAM-1. NKG2D associates with DAP10 adaptor protein which is containing a tyrosine-based signaling motif (YINM). The YINM is phosphorylated by Src family kinases and recruits p85 subunit of PI3K or Grb2. These interaction activates Vav1 and PLC- γ 2 that lead to the activation of Ca²⁺ mobilization and cytotoxicity (83). 2B4, NTB-A, and CRACC that belong to SLAM family receptors contain immunoreceptor tyrosine-based switch motif (ITSM) (84) which recruits, after phosphorylation, SAP, EAT-2, and ERT adaptor proteins (85). SAP binds Src family kinase Fyn, and this interaction leads to NK cell activation through phosphorylation of Vav1 and PLC- γ 2 (86, 87).

NK cells are equipped with diverse kinases, adaptors, and signaling modules depending on different activating receptors. Whereas, inhibition of signals from different activating receptors by a common ITIM-mediated mechanism suggests that there are critical signaling molecule as a convergent point. The redundancies of signaling molecules at each steps are different. Src family kinases, such as Lck, Fyn, Src, Lyn, Yes, and Fgr, are important molecules for most of activating receptors. But, no single kinase is indispensable as they are redundant for

NK cell activation (88). On the other hand, PLC- γ and Vav proteins are non-redundant for NK cell activation and play as a critical point for NK cell activation (82, 89). Therefore, the identification of signaling components play as critical checkpoints for NK cell activation can facilitate the understanding of cooperative mechanisms between coactivation receptors.

I-5. Signaling for synergistic activation of NK cells

NK cell activation is strictly regulated by the requirement for multiple engagement of coactivation receptors during target cells recognition (77, 90). The signals from specific combinations of coactivation receptors, such as NKG2D and 2B4, synergize to induce effective killing of target cells (77, 91). But, other combinations can make different effects, such as no increase over another activating signal or simple additive effect. Study of mechanisms how signals from disparate activating signals are integrated to produce potent effector functions of NK cells is a very difficult task because diverse activating receptors signals through different modules. Although NK cells are stimulated by synergistic combination of receptors, a single ITIM-containing inhibitory receptor stimulation is dominant over the synergy. This implies there are central checkpoints where signals from multiple receptors converge but inhibitory signal intersect.

Studies using NKG2D and 2B4 synergy model identified several checkpoints and molecular mechanisms integrating signals from multiple receptors (87, 92). The Vav1 as a central convergent point is advocated by the mechanism that Vav1 is an essential target of SHP-1 recruited to inhibitory receptors (93). Coengagement of NKG2D and 2B4 induces Vav1 phosphorylation and synergistic phosphorylation of PLC- γ 2, Ca²⁺ mobilization, and degranulation, while single receptor stimulation also induces Vav1 phosphorylation, but not the following events including effector functions of NK cells. This is explained by cell-intrinsic inhibition mechanism mediated by E3 ubiquitin ligase c-Cbl (87). The threshold inhibiting

Vav1-dependent downstream signal, that is formed by c-Cbl, is not overcome by a signal from single activating receptor, but by synergistic signal from certain combination of coactivation receptors.

Following study of NKG2D and 2B4 synergy unveiled another mechanism for synergistic activation (92). The extent of Vav1 phosphorylation by NKG2D and 2B4 combination is equivalent to the sum of phosphorylations induced by individual receptors. But, certain combinations of receptors do not enhance the phosphorylation of Vav1 over the extent induced by single receptor, even though each receptor stimulation independently induced phosphorylation of Vav1. This suggests a regulation mechanism that is upstream of Vav1. SLP-76 and LAT adaptor molecules are known as essential molecules for Vav1 signaling and signaling complexes formation in T cells (94). In NK cells, synergistic activation by NKG2D and 2B4 is largely dependent on SLP-76, but not LAT. Signals from each NKG2D and 2B4 induced phosphorylation of SLP-76 at disparate tyrosine residues, Tyr128 and Tyr113 respectively. Both phosphorylations at Tyr128 and Tyr113 are required for potent activation of Vav1 and subsequent synergistic NK cell activation. This mechanism appoints SLP-76 as an another checkpoint for permissive activation during synergy between activating receptors.

I-6. X-linked lymphoproliferative disease

X-linked lymphoproliferative disease (XLP) is a rare disease that has an incidence of 1~2 per million males (95). Key features of XLP are severe EBV-induced fulminant infectious mononucleosis (FIM), hemophagocytic lymphohistiocytosis (HLH), B-cell lymphoma, and dysgammaglobulinemia (96). Similar to other primary immunodeficiencies, HSCT is considered to be the only therapy to treat XLP (97). In 1998, *SH2D1A* gene encoding SAP was found as a molecular cause of the disease, and clues to how mutations of *SH2D1A*, which is associated with SLAM family receptors, contribute to disease (98). SAP associates with conserved ITSM motif in the cytoplasmic domain of SLAM via its SH2 domain. SAP competes with another SH2 containing protein, SHP-2 phosphatase, for binding to SLAM limiting its recruitment. SAP-deficient NK cells from XLP were found to have impaired cytotoxicity (99). Therefore, SAP-deficiency is known to render SLAM receptors inhibitory through the recruitment of phosphatases (100). But it was also reported that the inhibitory role of SAP-deficiency is not the case of NKG2D or DNAM-1 stimulation (101). Therefore, in NKG2D and 2B4 combination model, only activating role of 2B4 is to be considered. And NK cells from XLP patient can be a primary cell model to confirm mechanisms found in NKG2D and 2B4 costimulation of NK cell line.

I-7. Purpose of study and rationale for study design

The purpose of this study is to understand activating receptor signaling mechanisms of NK cells and provide theoretical basis in the establishment of activity-modulating strategies for NK cell-based therapy.

For this purpose,

1. Human NK cell line and primary NK cells, not mouse NK cells, were used for investigation.
2. NK cells were activated not only with single activating receptor stimulation but also with multiple activating receptor stimulation to mimic target cell recognition in physiological condition.
3. Specific activating receptors combination, that is NKG2D and 2B4, that induces synergistic pattern of NK cell activation was selected for activity-enhancing strategy.
4. Relatively less-known non-ITAM-associated activating receptors, not ITAM-associated receptors, were selected for investigation.
5. Critical checkpoint molecules of signal transduction were searched.
: Intrinsic inhibition point – target for activity-enhancing strategy
: Signal convergence point – criteria for selection of receptor combination
6. NK cells from XLP1 patient which is deficient in 2B4 receptor signaling were used to confirm newly found mechanism.
7. Mechanisms discovered from non-ITAM receptor model were verified in ITAM receptor model to confirm whether they are common mechanisms of NK cells.
8. Role of each signal from separate receptors at convergence point were investigated to explain how separate signals are integrated.
9. The level of changes between signal activation and following activity of NK cells were compared and checked for their coincidence.
10. Interplay among found signaling molecules were investigated.

I-8. References

1. Weinblatt, M. E. *et al.* Efficacy of low-dose methotrexate in rheumatoid arthritis. *N. Eng. J. Med.* **312**, 818-822 (1985).
2. Mumprecht, S., Matter, M., Pavelic, V. & Ochsenein, A. F. Imatinib mesylate selectively impairs expansion of memory cytotoxic T cells without affecting the control of primary viral infections. *Blood* **108**, 3406-3413 (2006).
3. Anand, P., Sundaram, C., Jhurani, S., Kunnumakkara, A. B. & Aggarwal, B. B. Curcumin and cancer: An “old-age” disease with an “age-old” solution. *Cancer Lett.* **267**, 133-164 (2008).
4. Zitvogel, L., Tesniere, A. & Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat. Rev. Immunol.* **6**, 715-727 (2006).
5. Sharma, P., Wagner, K., Wolchok, J. D. & Allison, J. P. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. *Nat. Rev. Cancer* **11**, 805-812 (2011).
6. Farkona, S., Diamandis, E. P. & Blasutig, I. M. Cancer immunotherapy: the beginning of the end of cancer? *BMC Med.* **14**, 73 (2016).
7. Zitvogel, L., Apetoh, L., Ghiringhelli, F. & Kroemer, G. Immunological aspects of cancer chemotherapy. *Nat. Rev. Immunol.* **8**, 59-73 (2008).
8. Ljunggren, H. G. & Malmberg, K.J. Prospects for the use of NK cells in

- immunotherapy of human cancer. *Nat. Rev. Immunol.* **7**, 329-339 (2007).
9. Ruggeri, L. *et al.* Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* **295**, 2097-2100 (2002).
 10. Ruggeri, L. *et al.* Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* **94**, 333-339 (1999).
 11. Shlomchik, W. D. *et al.* Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* **285**, 412-415 (1999).
 12. Miller, J. S. *et al.* Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood* **105**, 3051-3057 (2005).
 13. Klingemann, H. G. & Martinson, J. *Ex vivo* expansion of natural killer cells for clinical applications. *Cytotherapy* **6**, 15-22 (2004).
 14. Vacca, P. *et al.* NK cells and other innate lymphoid cells in hematopoietic stem cell transplantation. *Front. Immunol.* **7**, 188 (2016).
 15. Bernink, J. H. *et al.* Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat. Immunol.* **14**, 221-229 (2013).
 16. Seillet, C., Belz, G. T. & Huntington, N. D. Development, homeostasis, and heterogeneity of NK cells and ILC1. *Curr. Top. Microbiol. Immunol.* **395**, 37-61 (2015).
 17. Halim, T. Y. Group 2 innate lymphoid cells in disease. *Int. Immunol.* **28**, 13-22 (2016).
 18. Montaldo, E., Juelke, K. & Romagnani, C. Group 3 innate lymphoid cells

- (ILC3s): origin, differentiation, and plasticity in humans and mice. *Eur. J. Immunol.* **45**, 2171-2182 (2015).
19. Montaldo, E. *et al.* Human innate lymphoid cells. *Immunol. Lett.* (2016).
 20. Scoville, S. D. *et al.* A progenitor cell expressing transcription factor ROR γ t generates all human innate lymphoid cell subsets. *Immunity* **44**, 1140-1150 (2016).
 21. McKenzie, A. N., Spits, H. & Eberl, G. Innate lymphoid cells in inflammation and immunity. *Immunity* **41**, 366-374 (2014).
 22. Sonnenberg, G. F. & Artis, D. Innate lymphoid cells in the initiation, regulation and resolution of inflammation. *Nat. Med.* **21**, 698-708 (2015).
 23. Klose, C. S. *et al.* Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* **157**, 340-356 (2014).
 24. Fuchs, A. *et al.* Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN- γ -producing cells. *Immunity* **38**, 769-781 (2013).
 25. Trinchieri, G. Biology of natural killer cells. *Adv. Immunol.* **47**, 187-376 (1989).
 26. Herberman, R.B., Nunn, M.E., Holden, H.T., Lavrin, D.H. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors, II: characterization of effector cells. *Int. J. Cancer.* **16**, 230-239 (1975).
 27. Kiessling, R., Klein, E., Pross, H., Wigzell, H. "Natural" killer cells in the mouse, II: cytotoxic cells with specificity for mouse Moloney leukemia cells:

- characteristics of the killer cell. *Eur. J. Immunol.* **5**, 117-121 (1975).
28. Lanier, L.L., Phillips, J.H., Hackett, J.Jr., Tutt, M., Kumar, V. Natural killer cells: definition of a cell type rather than a function. *J. Immunol.* **137**, 2735-2739 (1986).
29. Ljunggren, H.G., Karre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today.* **11**, 237-244 (1990).
30. Karre, K., Ljunggren, H.G., Piontek, G. & Kiessling, R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defense strategy. *Nature* **319**, 675-678 (1986).
31. Vivier, E., Nunes, J.A. & Vely, F. Natural killer cell signaling pathways. *Science* **306**, 1517-1519 (2004).
32. Tomasello, E., Blery, M., Vely, F. & Vivier, E. Signaling pathways engaged by NK cell receptors: double concerto for activating receptors, inhibitory receptors and NK cells. *Semin. Immunol.* **12**, 139-147 (2000).
33. Lanier, L.L. Natural killer cell receptor signaling. *Curr. Opin. Immunol.* **15**, 308-314 (2003).
34. Lanier, L.L. NK cell recognition. *Annu. Rev. Immunol.* **23**, 225-274 (2005).
35. Long, E.O. Ready for prime time: NK cell priming by dendritic cells. *Immunity* **26**, 385-387 (2007).
36. Walzer, T., Dalod, M., Robbins, S.H., Zitvogel, L. & Vivier, E. Natural-killer cells and dendritic cells: "I" union fait la force." *Blood* **106**, 2252-2258 (2005).

37. Fehniger, T.A. *et al.* CD56^{bright} natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* **101**, 3052-3057 (2003).
38. Laouar, Y., Sutterwala, F.S., Gorelik, L. & Flavell, R.A. Transforming growth factor- β controls T helper type 1 cell development through regulation of natural killer cell interferon- γ . *Nat. Immunol.* **6**, 600-607 (2005).
39. Ghiringhelli, F. *et al.* CD4⁺CD25⁺ T regulatory cells inhibit natural killer cell functions in a transforming growth factor- β -dependent manner. *J. Exp. Med.* **202**, 1075-1085 (2005).
40. Moretta, L. *et al.* Effector and regulatory events during natural killer-dendritic cell interactions. *Immunol. Rev.* **214**, 219-228 (2006).
41. Martin-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN- γ for T_H1 priming. *Nat. Immunol.* **5**, 1260-1265 (2004).
42. Stewart, C.A. & Vivier, E. Strategies of NK cell recognition and their roles in tumor immunosurveillance. in *How the Immune System Recognizes Self and Nonself: Immunoreceptors and Their Signaling* (ed. Kitamura, D.) 37-81 (Springer, Tokyo, 2007).
43. Diefenbach, A., Jensen, E.R., Jamieson, A.M. & Raulet, D.H. Rae1 and H60 ligands of the NKG2D receptor stimulate tumor immunity. *Nature* **413**, 165-171 (2001).
44. Smyth, M.J. *et al.* NKG2D function protects the host from tumor initiation. *J. Exp. Med.* **202**, 583-588 (2005).

45. Orange, J.S. Human natural killer cell deficiencies. *Curr. Opin. Allergy Clin. Immunol.* **6**, 399-409 (2006).
46. Imai, K., Matsuyama, S., Miyake, S., Suga, K. & Nakachi, K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* **356**, 1795-1799 (2000).
47. Ruggeri, L., Aversa, F., Martelli, M.F. & Velardi, A. Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. *Immunol. Rev.* **214**, 202-218 (2006).
48. Koh, C.Y. *et al.* Augmentation of antitumor effects NK cell inhibitory receptor blockade in vitro and in vivo. *Blood* **97**, 3132-3137 (2001).
49. Lee, S.H., Miyagi, T. & Biron, C.A. Keeping NK cells in highly regulated antiviral warfare. *Trends Immunol.* **28**, 252–259 (2007).
50. Scalzo, A.A., Corbett, A.J., Rawlinson, W.D., Scott, G.M. & Degli-Esposti, M.A. The interplay between host and viral factors in shaping the outcome of cytomegalovirus infection. *Immunol. Cell Biol.* **85**, 46–54 (2007).
51. Smith, H.R. *et al.* Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc. Natl. Acad. Sci. USA* **99**, 8826–8831 (2002).
52. Arase, H., Mocarski, E.S., Campbell, A.E., Hill, A.B. & Lanier, L.L. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* **296**, 1323–1326 (2002).

53. Vidal, S.M. & Lanier, L.L. NK cell recognition of mouse cytomegalovirus-infected cells. *Curr. Top. Microbiol. Immunol.* **298**, 183–206 (2006).
54. Raulet, D.H. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat. Immunol.* **5**, 996–1002 (2004).
55. Paya, C.V., Patick, A.K., Leibson, P.J. & Rodriguez, M. Role of natural killer cells as immune effectors in encephalitis and demyelination induced by Theiler's virus. *J. Immunol.* **143**, 95–102 (1989).
56. Fairweather, D., Kaya, Z., Shellam, G.R., Lawson, C.M. & Rose, N.R. From infection to autoimmunity. *J. Autoimmun.* **16**, 175–186 (2001).
57. Robbins, S.H. *et al.* Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog.* **3**, e123 (2007).
58. van Dommelen, S.L.H. *et al.* Perforin and granzymes have distinct roles in defensive immunity and immunopathology. *Immunity* **25**, 835–848 (2006).
59. Moretta, L. *et al.* Effector and regulatory events during natural killer-dendritic cell interactions. *Immunol. Rev.* **214**, 219–228 (2006).
60. Piccioli, D., Sbrana, S., Melandri, E. & Valiante, N.M. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J. Exp. Med.* **195**, 335–341 (2002).

61. Hayakawa, Y. *et al.* NK cell TRAIL eliminates immature dendritic cells in vivo and limits dendritic cell vaccination efficacy. *J. Immunol.* **172**, 123–129 (2004).
62. Degli-Esposti, M.A. & Smyth, M.J. Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nat. Rev. Immunol.* **5**, 112–124 (2005).
63. Martin-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN- γ for TH1 priming. *Nat. Immunol.* **5**, 1260–1265 (2004).
64. Lu, L. *et al.* Regulation of activated CD4⁺ T cells by NK cells via the Qa-1–NKG2A inhibitory pathway. *Immunity* **26**, 593–604 (2007).
65. Takeda, K. & Dennert, G. The development of autoimmunity in C57BL/6 lpr mice correlates with the disappearance of natural killer type 1-positive cells: evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. *J. Exp. Med.* **177**, 155–164 (1993).
66. Anne Croy, B., van den Heuvel, M.J., Borzychowski, A.M. & Tayade, C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol. Rev.* **214**, 161–185 (2006).
67. Hanna, J. *et al.* Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat. Med.* **12**, 1065–1074 (2006).

68. Burshtyn, D.N. *et al.* Recruitment of tyrosine phosphatase HCP (SHP-1) by the killer cell inhibitor receptor. *Immunity* **4**, 77-85 (1996).
69. Long, E.O. Negative signaling by inhibitory receptors: The NK cell paradigm. *Immunol. Rev.* **224**, 70-84 (2008).
70. Yu, M.C. *et al.* An essential function for beta-arrestin 2 in the inhibitory signaling of natural killer cells. *Nat. Immunol.* **9**, 898-907 (2008).
71. Endt, J. *et al.* Inhibitory receptor signals suppress ligation-induced recruitment of NKG2D to GM1-rich membrane domains at the human NK cell immune synapse. *J. Immunol.* **178**, 5606-5611 (2007).
72. Watzl, C. & Long, E.O. Natural killer cell inhibitory receptors block actin cytoskeleton-dependent recruitment of 2B4 (CD244) to lipid rafts. *J. Exp. Med.* **197**, 77-85 (2003).
73. Peterson, M.E. & Long, E.O. Inhibitory receptor signaling via tyrosine phosphorylation of the adaptor Crk. *Immunity* **29**, 578-588 (2008).
74. Anfossi, N. *et al.* Human NK cell education by inhibitory receptors for MHC class I. *Immunity* **25**, 331-342 (2006).
75. Gasser, S. & Raulet, D.H. Activation and self-tolerance of natural killer cells. *Immunol. Rev.* **214**, 130-142 (2006).
76. Yokoyama, W.M. & Kim, S. Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunol. Rev.* **214**, 143-154 (2006).
77. Bryceson, Y.T., March, M.E., Ljunggren, H.G. & Long, E.O. Activation,

- coactivation, and costimulation of resting human natural killer cells. *Immunol. Rev.* **214**, 73-91 (2006).
78. Bottino, C., Castriconi, R., Moretta, L. & Moretta, A. Cellular ligands of activating NK receptors. *Trends Immunol.* **26**, 221-226 (2005).
79. Gasser, S. & Raulet, D.H. Activation and self-tolerance of natural killer cells. *Immunol. Rev.* **214**, 130-142 (2006).
80. Bryceson, Y.T., March, M.E., Ljunggren, H.G. & Long, E.O. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107**, 159-166 (2006).
81. Ting, A.T., Karnitz, T.L., Schoon, R.A., Abraham, R.T. & Leibson, P.J. Fc gamma receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-gamma 1 and PLC-gamma 2 in natural killer cells. *J. Exp. Med.* **176**, 1751-1755 (1992).
82. Cella, M. *et al.* Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. *J. Exp. Med.* **200**, 817-823 (2004).
83. Upshaw, J.L. *et al.* NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat. Immunol.* **7**, 524-532 (2006).
84. Claus, M., Meinke, S., Bhat, R. & Watzl, C. Regulation of NK cell activity by 2B4, NTB-A and CRACC. *Front. Biosci.* **13**, 956-965 (2008).
85. Sayos, J. *et al.* The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* **395**, 462-469

- (1998).
86. Latour, S. *et al.* Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nat. Cell Biol.* **5**, 149-154 (2003).
 87. Kim, H.S., Das, A., Gross, C.C., Bryceson, Y.T. & Long, E.O. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity* **32**, 175-186 (2010).
 88. van Oers, N.S., Lowin-Kropf, B., Finlay, D., Connolly, K. & Weiss, A. alpha beta T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. *Immunity* **5**, 429-436 (1996).
 89. Caraux, A. *et al.* Phospholipase C-gamma2 is essential for NK cell cytotoxicity and innate immunity to malignant and virally infected cells. *Blood* **107**, 994-1002 (2006).
 90. Bryceson, Y.T., Ljunggren, H.G. & Long E.O. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. *Blood* **114**, 2657-2666 (2009).
 91. Lanier, L.L., Corliss, B. & Phillips, J.H. Arousal and inhibition of human NK cells. *Immunol. Rev.* **155**, 145-154 (1997).
 92. Kim, H.S. & Long, E.O. Complementary phosphorylation sites in the adaptor protein SLP-76 promote synergistic activation of natural killer cells. *Sci. Signal.* **5**, ra49 (2012).
 93. Stebbins, C. *et al.* Vav1 dephosphorylation by the tyrosine phosphatase SHP-1

- as a mechanism for inhibition of cellular cytotoxicity. *Mol. Cell. Biol.* **23**, 6291-6299 (2003).
94. Koretzky, G.A., Abtahian, F. & Silverman, M.A. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat. Rev. Immunol.* **6**, 67-78 (2006).
95. Tangye, S. G. XLP: Clinical features and Molecular Etiology due to Mutations in *SH2D1A* encoding SAP. *J. Clin. Immunol.* **34**, 772-779 (2014).
96. Booth, C. *et al.* X-linked lymphoproliferative disease due to SAP/SH2D1A deficiency: a multicenter study on the manifestations, management and outcome of the disease. *Blood* **117**, 53-62 (2011).
97. Lankester, A. C. *et al.* Allogeneic stem cell transplantation in X-linked lymphoproliferative disease: two cases in one family and review of the literature. *Bone Marrow Transplant.* **36**, 99-105 (2005).
98. Sayos, J. *et al.* The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* **395**, 462-469 (1998).
99. Bottino, C. *et al.* NTB-A, a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. *J. Exp. Med.* **194**, 235-246 (2001).
100. Dong, Z. *et al.* The adaptor SAP controls NK cell activation by regulating the enzymes Vav-1 and SHIP-1 and by enhancing conjugates with target cells.

Immunity **36**, 974-985 (2012).

101. Meazza, R. *et al.* XLP1 inhibitory effect by 2B4 does not affect DNAM-1 and NKG2D activating pathways in NK cells. *Eur. J. Immunol.* **44**, 1526-1534 (2014).

Chapter II.
**NK cell function triggered
by multiple activating receptors
is negatively regulated
by glycogen synthase kinase-3 β**

**Published in
Cellular Signalling 27 (2015) 1731-1741**

II-1. Abstract

Activation of NK cells is triggered by combined signals from multiple activating receptors that belong to different families. Several NK cell activating receptors have been identified, but their role in the regulation of effector functions is primarily understood in the context of their individual engagement. Therefore, little is known about the signaling pathways broadly implicated by the multiple NK cell activation cues. Here we provide evidence pointing to glycogen synthase kinase (GSK)-3 β as a negative regulator of multiple NK cell activating signals. Using an activation model that combines NKG2D and 2B4 and tests different signaling molecules, we found that GSK-3 undergoes inhibitory phosphorylation at regulatory serine residues by the engagement of NKG2D and 2B4, either individually or in combination. The extent of such phosphorylation was closely correlated with the degree of NK cell activation. NK cell functions, such as cytokine production and cytotoxicity, were consistently enhanced by the knockdown of GSK-3 β or its inhibition with different pharmacological inhibitors, whereas inhibition of the GSK-3 α isoform had no effect. In addition, NK cell function was augmented by the overexpression of a catalytically inactive form of GSK-3 β . Importantly, the regulation of NK cell function by GSK-3 β was common to diverse activating receptors that signal through both ITAM and non-ITAM pathways. Thus, our results suggest that GSK-3 β negatively regulates NK cell activation

and that modulation of GSK-3 β function could be used to enhance NK cell activation.

II-2. Introduction

NK cells are a type of cytotoxic lymphocytes that contribute critically to host defense against viral infection and cellular transformation and to the regulation of immune responses (1, 2). Such functions rely on the intrinsic capacity of NK cells to kill virally-infected, stressed and transformed cells and to produce a variety of cytokines (e.g., IFN- γ , TNF- α) and chemokines (e.g., MIP-1 α/β) without prior immunization. These two major NK cell functions can be rapidly induced by multiple germline-encoded activating receptors that recognize ligands preferentially expressed on abnormal cells (1, 3, 4). Without the help of cytokine stimulation, NK cell activation is tightly regulated by the requirement for the engagement by target cells of multiple co-activating receptors, each with its unique ligand specificity and signaling properties (5, 6). Synergy among different activating receptors, such as combination of NKG2D (CD314) and 2B4 (CD244), is required to trigger efficient cytotoxicity and cytokine production by resting NK cells, which is different from the requirement for the activation of cytokine-stimulated NK cells (1, 5, 7-9). Thus, signaling pathways for the activation of NK cells are often regulated by combined signals from multiple activating receptors, which can operate in synergy.

Some signaling molecules implicated in the regulation of NK cell functions have been uncovered. However, it has been challenging to identify critical signaling molecules involved in NK cell activation by multiple activating receptors as NK cells use diverse signaling modules depending on the engagement of different activating receptors (10, 11). Two signaling molecules that are critical and non-redundant for

the activation of NK cells are Vav and PLC- γ proteins (1). Their deficiency is accompanied by severe defects in NK cell functions triggered by multiple activating receptors (12-16). In this regard, identifying signaling molecule(s) that are broadly implicated in multiple activation pathways and their contribution to NK cell functions is important to understand the consequence of target cell recognition by NK cells.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase that functions as a convergent point of diverse signaling pathways and regulates many cellular processes such as metabolism, differentiation, proliferation, apoptosis, and immune responses (17, 18). Accordingly, GSK-3 is implicated in the pathogenesis of various diseases and has emerged as a therapeutic target for the treatment of cancer, inflammation, diabetes, and neurodegenerative diseases (19), (20, 21). GSK-3 has two isoforms encoded by different genes, GSK-3 α and GSK-3 β , which share high sequence homology within the kinase domain but differ substantially in the N- and C-terminal regions (22). Unlike many other kinases, GSK-3 is constitutively active in resting cells and inhibited by various kinases (e.g. Akt, RSK) in response to diverse stimuli through phosphorylation at Ser21 in GSK-3 α and at Ser9 in GSK-3 β (18, 23). Although these isoforms compensate for each other in certain cellular functions (24), they also have unique and non-overlapping roles as evidenced by distinct phenotypes observed in GSK-3 α and GSK-3 β null mice (25-27).

GSK-3 has gained increasing attention as a critical regulator of many components of innate and adaptive immune system, largely due to its modulation of crucial transcription factors (17). Inhibition of GSK-3 by pharmacological agents

and molecular manipulation (e.g., genetic deficiency or knockdown) greatly but not uniformly influences the inflammatory cytokine and chemokine repertoire triggered by diverse stimuli in monocytes, dendritic cells, mast cells, and T cells (28-33). These studies indicate that immune cell functions affected by GSK-3 appear to be diverse and context-dependent according to the types of stimuli and immune cells. To date, the role of GSK-3 in NK cell functions remains largely unclear, given the results of a previous study using a single pharmacological agent for GSK-3 inhibition and in the context of NK cell stimulation via target cells in combination with 2B4 (34). NK cells have a multiplicity of receptors with different signaling properties for activation (10, 11, 35): receptors associated with ITAM-bearing molecules (e.g., CD16, NKp30), the DAP10-associated receptor NKG2D, receptors of the signaling lymphocytic activation molecule (SLAM) family (e.g., 2B4), and other activating receptors (e.g., DNAM-1).

In this study, we investigated the effect of GSK-3 modulation on NK cell functions after engagement of defined and diverse activating receptors alone or in combination. Our results showed that GSK-3, specifically GSK-3 β , negatively regulates NK cell responses such as cytokine production and cytotoxicity induced by multiple activating receptors as well as by sensitive target cells. We also found that such negative regulation by GSK-3 β is dependent on its kinase activity, thus suggesting the inhibition of GSK-3 β as a potential strategy to augment NK cell activation.

II-3. Materials and Methods

II-3-1. Cell lines and reagents

Human blood samples were drawn from healthy donors for research purposes under a protocol approved by the Asan Medical Center Institutional Review Board with informed consent. PBMCs were isolated using lymphocyte separation medium (MP Biomedicals). Human NK cells were isolated from PBMCs by negative selection with an NK cell isolation kit (StemCell Technologies) as described (36). Isolated cell populations were 97 to 99% CD3[−]CD56⁺ as assessed by flow cytometry. PBMCs or NK cells were used for functional assays after 24 h incubation with human recombinant IL-2 (rIL-2) (200 U/ml; Roche). The human NK cell line NKL (a gift of M. Robertson, Indiana University) was cultured in RPMI1640 (Corning) supplemented with 10% FBS, 1 mM sodium pyruvate, and 200 U/ml rIL-2. NK92 cell line transduced with 176 V variant (high affinity) of CD16 (CD16[176 V]. NK-92; a gift of K.S. Campbell, Fox Chase Cancer Center) (37, 38) was cultured in α -MEM (Gibco) supplemented with 20% FBS, 1% MEM vitamin solution (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), and 200 U/ml rIL-2. P815 and K562 cells were cultured in IMDM (Corning) supplemented with 10% FBS and 2 mM l-glutamine. Plat-A retroviral packaging cell line (Cell Biolabs) was cultured in DMEM (Corning) supplemented with 10% FBS, 1 μ g/ml puromycin, and 10 μ g/ml blasticidin. All chemicals were from Calbiochem unless indicated otherwise.

II-3-2. NK cell expansion

Primary human NK cells were expanded as previously described (39), with some modifications. PBMCs (1.5×10^6) were incubated in a 24-well tissue culture plate with 100 Gy-irradiated K562-mb15-41BBL cells (a gift of D. Campana, National University of Singapore) (1×10^6) in Stem Cell Growth Medium (SCGM; CellGenix) supplemented with 10% FBS and 10 U/ml rIL-2. The medium was exchanged every 2 days with fresh medium with rIL-2. After 1 week, residual T cells were depleted with a CD3 positive selection kit (StemCell Technologies). Purified NK cells were incubated in SCGM supplemented with 10% FBS, 100 U/ml rIL-2, and 5 ng/ml rIL-15 for two additional weeks with a medium exchange being made every 2 days. The expanded cell populations were 96 to 99% CD3[−]CD56⁺ as assessed by flow cytometry.

II-3-3. Antibodies (Abs)

Abs for the detection of these proteins were obtained from the sources indicated: NKG2D (149810; R&D Systems); CD244/2B4 (C1.7; Beckman Coulter); CD337/NKp30 (P30-15; BioLegend); isotype control mouse IgG1 (MOPC-21; Sigma); c-Cbl (7G10; Millipore); CD16 (3G8), CD94 (HP-3D9), NFAT1 (1/NFAT-1), and actin (C4) (BD Biosciences); PLC γ 2 (Q-20; Santa Cruz); pS21/9-GSK-3 α / β (9331), pS9-GSK-3 β (9336), GSK-3 α / β (5676), GSK-3 β (9315), pS33/37/T41- β -Catenin (9561), pY1217-PLC γ 2 (3871), pS473-Akt (9271), Akt (9272), p-ERK1/2 (9101), ERK1/2 (9102), and Vav1 (2153) (Cell Signaling). Following fluorochrome-conjugated antibodies were used for flow cytometric analysis: anti-CD3-PerCP (SK7), anti-CD56-PE (NCAM16.2), anti-CD107a-FITC (H4A3), and anti-IFN- γ -FITC (25723.11) (BD Biosciences). Anti-mouse and rabbit antibodies conjugated

with horseradish peroxidase (HRP) were from Santa Cruz Biotechnology.

II-3-4. Flow cytometric analysis of NK cell activation

PBMCs or NK cells were stimulated by incubation with an equal number of K562 or P815 cells labeled with receptor-specific Abs. For degranulation assay, cell surface expression of CD107a was measured by flow cytometry after 2 h incubation as described (40). For intracellular cytokine assay, brefeldin A and monensin were added after 1 h of incubation, after which intracellular IFN- γ expression was measured after an additional 5 h incubation using flow cytometry as described (41, 42). For PBMCs, lymphocytes were gated on forward/side scatter, and CD3-CD56⁺ NK cells were analyzed (42).

II-3-5. Cell stimulation

For Ab-mediated crosslinking of NK receptors, NK cells were incubated with 10 $\mu\text{g/ml}$ isotype control mAb or mAbs specific for NK receptors for 30 min on ice. After washing unbound Abs, cells were stimulated by crosslinking with 30 $\mu\text{g/ml}$ goat anti-mouse F(ab')₂ at 37 °C for the indicated times. For bead-mediated stimulation, goat anti-mouse IgG-coated Dynabeads (Invitrogen) were incubated with 3 μg Ab/ 4×10^7 beads specific for NK receptors for 1 h at 4 °C with rotation. Cells were stimulated with Ab-labeled beads at the ratio of 1:8 (cell:bead) for 3 h (RT-PCR) or 12 h (cytokine release assay).

II-3-6. Measurement of cytokine secretion

IFN- γ (Pierce) and MIP-1 α (R&D Systems) secreted from NK cells after stimulation with beads coated with mAbs specific for NK receptors were measured by enzyme-linked immunosorbent assay (ELISA) as described (43).

II-3-7. Immunoblot analysis

Stimulated NK cells were washed with ice-cold PBS and lysed in a lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM NaVO₃, 50 mM NaF, 1 mM PMSF, and protease inhibitor cocktail [Roche]) for 30 min on ice. Cell debris including nuclei was removed by centrifugation, and supernatants were recovered. Protein concentration in cell lysates was determined with a Micro BCA protein assay kit (Pierce). Lysates were diluted with 4 \times NuPAGE LDS sample buffer (Invitrogen) containing 50 mM DTT. Equal amounts of protein from each sample were resolved on 8% SDS-PAGE gel and transferred onto PVDF membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol). Membranes were blocked with 5% skim milk in TBS-T (TBS with 0.1% Tween 20) for 1 h at room temperature (RT), incubated with primary Ab overnight at 4 $^{\circ}$ C, and then incubated with HRP-conjugated secondary Ab for 1 h at RT. Blots were developed with SuperSignal West Pico (Pierce) and signals were detected with LAS-4000 (Fujifilm).

II-3-8. RNA interference

NKL cells were transfected with 300 pmol of siRNAs with the Amaxa Nucleofector II system (Lonza). A total of 2 \times 10⁶ cells were resuspended in 100 μ l of Amaxa kit solution V, mixed with siRNA, and transfected with program O-017.

For a total of 48 h incubation at 37 °C, cells were rested for the last 24 h and then assayed as indicated. For knockdown in primary NK cells, 1.5×10^6 expanded cells were resuspended in 100 μ l of Amaxa kit solution for human macrophage, mixed with 300 pmol of siRNA, and transfected with program X-001. Thereafter, cells were incubated for 36 h in the presence of 200 U/ml rIL-2, rested for 12 h, and then assayed as indicated. For knockdown in CD16.NK92 cell line, 1.2×10^6 cells were resuspended in 100 μ l of Amaxa kit solution R, mixed with 300 pmol of siRNA, and transfected with program A-024. Cells were incubated for 48 h at 37 °C and then assayed as indicated. The siRNAs specific for GSK-3 α and GSK-3 β were obtained from IDT with the following sequences: GSK-3 α , 5'-CAA UAU UGU GAG GCU GAG AUA CUT T-3' (Sense) and 5'-AAA GUA UCU CAG CCU CAC AAU AUU GCA-3' (Antisense); GSK-3 β , 5'-AAG AAU CGA GAG CUC CAG AUC AUG A-3' (Sense) and 5'-UCA UGA UCU GGA GCU CUC GAU UCU UAA-3' (Antisense). The siRNAs specific for c-Cbl and Vav1 were described previously (7) and their sequences are as follows: c-Cbl, 5'-CCU CUC UUC CAA GCA CUG AdTdT-3' (Sense) and 5'-UCA GUG CUU GGA AGA GAG GdTdT-3' (Antisense); Vav1, 5'-CGU CGA GGU CAA GCA CAU UdTdT-3' (Sense) and 5'-AAU GUG CUU GAC CUC GAC GdTdT-3' (Antisense). The negative siRNA control was obtained from IDT and Dharmacon.

II-3-9. RT-PCR

To assess the expression of cytokines in NK cells, total RNA was isolated with RNeasy kit (QIAGEN). cDNA was synthesized from 1 μ g of RNA with ReverTra Ace qPCR RT kit (Toyobo) according to the manufacturer's instructions.

The following PCR primers were used: 5'-GCT GAC TAC TTT GAG ACG AGC-3' (Forward) and 5'-CCA GTC CAT AGA AGA GGT AGC-3' (Reverse) for human MIP-1 α ; 5'-CCA AAC CAA AAG AAG CAA GC-3' (Forward) and 5'-AGA AAC AGT GAC AGT GGA CC-3' (Reverse) for human MIP-1 β ; 5'-GCA TCG TTT TGG GTT CTC TTG GCT GTT ACT GC-3' (Forward) and 5'-CTC CTT TTT CGC TTC CCT GTT TTA GCT GCT GG-3' (Reverse) for human IFN- γ ; 5'-ACT CCA TCA TGA AGT GTG ACG-3' (Forward) and 5'-CAT ACT CCT GCT TGC TGA TCC-3' (Reverse) for human β -actin.

II-3-10. Cytotoxicity assay

For europium-based cytotoxicity assay, P815 cells were loaded with 40 μ M BATDA (Perkin Elmer) for 30 min at 37 $^{\circ}$ C. Cells were then washed in a medium with 1 mM sulfinpyrazone (Sigma), resuspended at 1×10^6 cells/ml in the medium, and incubated for 30 min at room temperature with mAbs (10 μ g/ml) to NK receptors. Cells were washed and incubated with effector cells in the presence of sulfinpyrazone for 2 h at 37 $^{\circ}$ C. Plates were mixed briefly and centrifuged at $400 \times g$ for 3 min. Supernatant (20 μ l) was incubated with 200 μ l of 20% europium solution (Perkin Elmer) in 0.3 M acetic acid for 5 min and detected with VICTOR X4 multilabel plate reader (Perkin Elmer).

II-3-11. Flow cytometric analysis of Ca²⁺ mobilization

NK cells were labeled for 30 min at 30 $^{\circ}$ C with dye-loading buffer (HBSS with 1% FBS, 4 μ g/ml Fluo-4 AM, and 4 mM probenecid). For experiments involving retroviral transduction of GSK-3 β mutant constructs co-expressing GFP,

NK cells were labeled for 30 min at 30 °C in HBSS with 1% FBS with Asante Calcium Red-AM (6 µg/ml, TEFLabs) and 4 mM probenecid. Cells were washed twice, resuspended in HBSS with 1% FBS, and incubated with mAbs (10 µg/ml) specific for NK receptors for 30 min on ice. Cells were resuspended in HBSS with 1% FBS, warmed for 5 min at 37 °C in a water bath and placed on a flow cytometer. After 30 s of data acquisition, 4 µg of crosslinking goat anti-mouse F(ab')₂ was added and events were acquired for a further 5 min. Data were analyzed with FlowJo software (Tree Star).

II-3-12. Retroviral transduction

To generate stable NKL cell line expressing GSK-3β mutants (wild-type [WT], constitutively active [CA; S9A], kinase dead [KD; K85A]), Plat-A retroviral packaging cell line was transfected with GFP retroviral vectors (pMX-IRES-GFP) that allows co-expression of cloned GSK-3β and GFP using X-tremeGENE 9 (Roche). Medium was changed after 24 h and virus-containing supernatant was collected after another 24 h. Virus-containing supernatant was mixed with fresh medium at 1:1 ratio, and 10 µg/ml polybrene and 200 U/ml rIL-2 was added to make transducing mix. 0.5×10^6 NKL cells for transduction were resuspended in 2.4 ml of transducing mix and then transferred to 12-well plate. The plate was centrifuged at 700 ×g for 30 min at 32 °C and incubated for 3 h at CO₂ incubator. After another centrifugation at 700 ×g for 30 min at 32 °C and 6 h incubation, cells were washed and resuspended with fresh medium. Three days after infection, cells with a matched level of GFP expression were selected by a FACS Aria cell sorter.

II-3-13. Statistical analysis

Each graph was generated from at least three independent experiments. Individual data points between two groups were analyzed by two-tailed Student's t test using the GraphPad Prism software.

II-4. Results

II-4-1. Effector functions of NK cells are enhanced by pharmacological inhibition of GSK-3

In an effort to identify signaling molecules broadly implicated in multiple activation pathways in NK cells, we used a model of NK cell activation that relies on combined signals from different receptors such as NKG2D and 2B4. Because NK cell activation is largely dependent on protein phosphorylation, we used a human phospho-kinase immunoblot array to probe the changes in phosphorylation events after stimulation of NKL cells with NKG2D and 2B4, either alone or in combination. Among the phospho-proteins analyzed, GSK-3 was chosen for further study based on the findings that both receptors induced GSK-3 phosphorylation and that pharmacological inhibition of GSK-3 modulated NK cell function (data not shown). As regulation of cytokine production by GSK-3 is common to various types of immune cells (17), we first assessed the role of GSK-3 in proinflammatory cytokine production by peripheral blood NK cells. GSK-3 was blocked by three different classes of GSK-3 inhibitors (44): lithium chloride (LiCl; small metal cations), a thiadiazolidinone (TDZD-8; non-ATP-competitive), and 6-bromoindirubin-3'-oxime (BIO; ATP-competitive). PBMCs were isolated from healthy donors, stimulated with IL-2 for 24 h, and incubated with K562 target cells for 6 h after pretreatment with each GSK-3 inhibitor for 1 h. The intracellular expression of IFN- γ was measured in CD3⁺ CD56⁺ NK cells among size-gated lymphocytes. As shown in Fig. 1A and B, pharmacological blockade of GSK-3 by all three inhibitors consistently increased

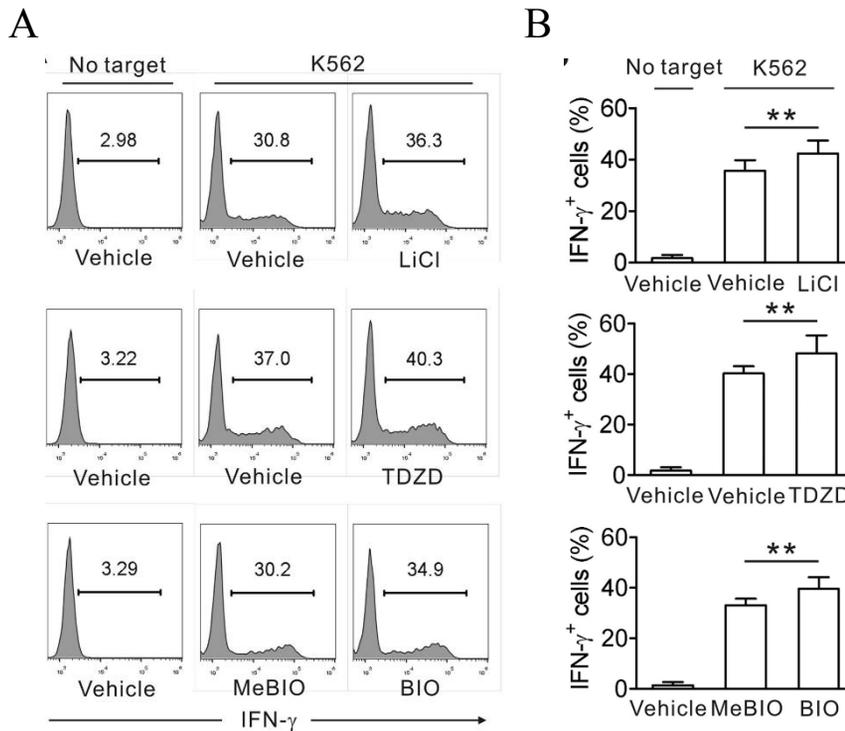


Fig. 1. GSK-3 regulates IFN- γ production by primary NK cells stimulated with target cell.

(A and B) PBMCs exposed to IL-2 were pretreated with GSK-3 inhibitor LiCl (5 mM), TDZD-8 (0.5 μ M), BIO (200 nM), or MeBIO (200 nM), an inactive control analog of BIO, for 1 h and were then mixed with K562 cells in the presence of inhibitor for intracellular cytokine assay. After 6 h incubation at 37 $^{\circ}$ C, cells were stained with fluorochrome-conjugated anti-CD3 and anti-CD56 mAbs for surface staining. Cytokine production by NK cells was measured in CD3 – CD56 + cells by flow cytometry after intracellular staining of IFN- γ . Representative FACS profiles (A) and graphs of statistical bar charts (B) showing expression of IFN- γ by NK cells. Error bars represent the SD. ** $P < 0.01$.

NK cell expression of IFN- γ , an observation compatible with that of a previous study using TDZD-8 as a single inhibitor (34). Similarly, GSK-3 inhibition also enhanced cytotoxic degranulation (indicated by CD107a positivity on NK cells) by NK cells upon stimulation with K562 target cells (Fig. 2).

We next investigated the functional significance of GSK-3 in the context of stimulation through defined receptors for further mechanistic study. Stimulation of either NKG2D or 2B4 alone induced modest and weak expression of IFN- γ by NK cells (data not shown). In comparison, stimulation through combination of NKG2D and 2B4 induced an apparent expression of IFN- γ by NK cells, which was significantly enhanced in the presence of GSK-3 inhibitors (Fig. 3A and B). Although the degree to which GSK-3 inhibition enhanced NK cell function varied by donor, it was consistently observed. The regulation of cytokine production by GSK-3 was also examined in NKL cells, a model human NK cell line for studying NK cell activation by NKG2D and 2B4 synergy. As observed in primary NK cells (Fig. 1A and B), production of cytokine IFN- γ and chemokine MIP-1 α by NKL cells after stimulation through combination of NKG2D and 2B4 was significantly increased by GSK-3 inhibition (Fig. 4). GSK-3 inhibitors tested did not significantly affect the viability of primary NK cells and NKL cell line, as assessed by annexin-V/PI staining (data not shown), thus ruling out their cytotoxic effect on NK cells during the time frame of cytokine assay. Collectively, these results indicate that GSK-3 plays an inhibitory role in NK cell cytokine production and cytotoxicity.

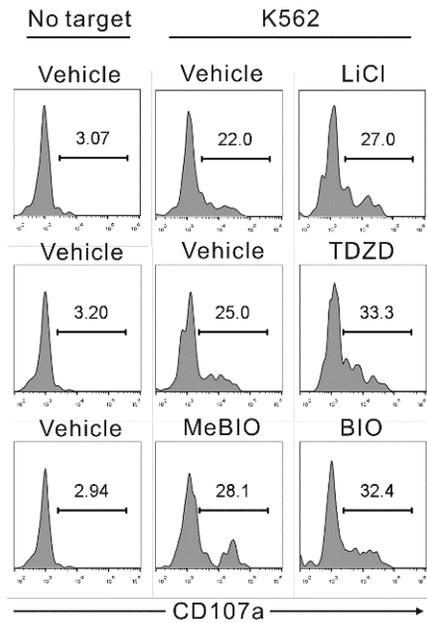


Fig. 2. GSK-3 regulates degranulation of primary NK cells stimulated with target cell.

PBMCs were pretreated with GSK-3 inhibitor LiCl (5 mM), TDZD-8 (0.5 μ M), BIO (200 nM), or MeBIO (200 nM), an inactive control analog of BIO, for 1 hr and then mixed with K562 cells in the presence of inhibitor and of fluorochrome-conjugated anti-CD107a mAb for degranulation assay. After 2 hr incubation at 37°C, cells were stained with fluorochrome-conjugated mAbs specific for CD3 and CD56 for surface staining. The percentage of CD107a⁺ cells was measured in CD3-CD56⁺ NK cells by flow cytometry. Data are representative of at least three independent experiments.

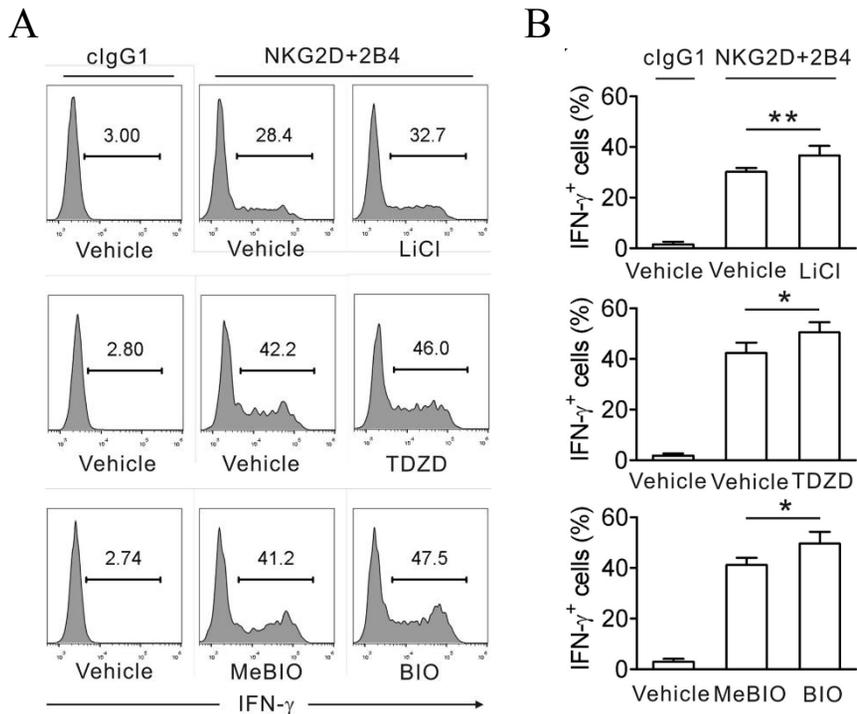


Fig. 3. GSK-3 regulates IFN- γ production by primary NK cells stimulated with synergistic activating receptor combination.

(A and B) PBMCs exposed to IL-2 were pretreated with GSK-3 inhibitor as described in Fig. 1A and were then mixed with P815 cells preincubated with isotype control mAb (cIgG1) or mAbs to NKG2D and 2B4 in the presence of inhibitor for intracellular cytokine assay. After 6 h incubation, cells were analyzed by flow cytometry as described in Fig. 1A for IFN- γ production by NK cells. Representative FACS profiles (A) and graphs of statistical bar charts (B) showing expression of IFN- γ by NK cells. Error bars represent the SD. *P < 0.05; **P < 0.01.

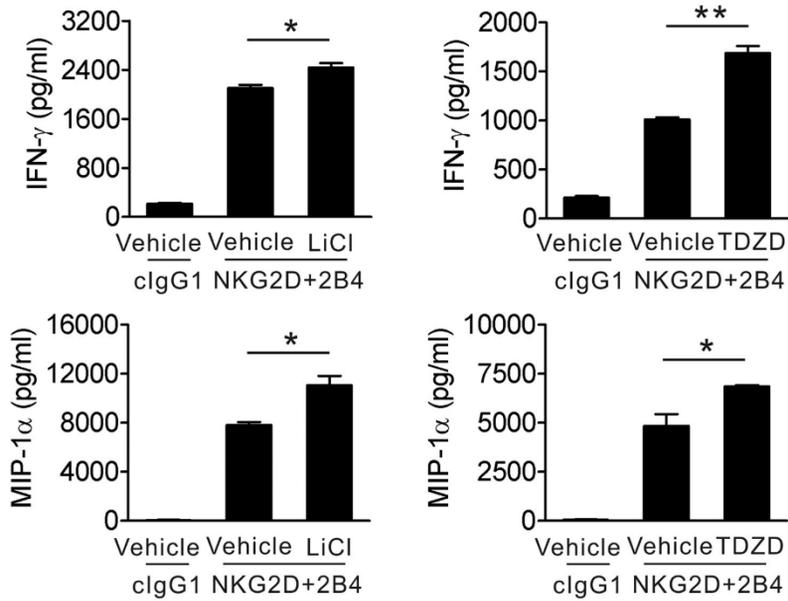


Fig. 4. GSK-3 regulates IFN- γ production by NKL cells stimulated with synergistic activating receptor combination.

Rested NKL cells were pretreated with GSK-3 inhibitor LiCl (5 mM) or TDZD-8 (0.5 μ M) for 1 h and then stimulated with beads coated with isotype control mAb (cIgG1) or mAbs specific for NKG2D and 2B4 in the presence of inhibitor. After 12 h incubation, IFN- γ and MIP-1 α in the supernatants were measured by ELISA. Values represent mean \pm SD. Data are representative of at least three independent experiments. *P < 0.05; **P < 0.01.

II-4-2. GSK-3 phosphorylation correlates with the activation status of NK cells

To study the regulation mechanism of GSK-3 during NK cell activation, we investigated whether stimulation of NK cells through NKG2D and 2B4 modulated the phosphorylation of GSK-3. The inhibitory phosphorylation at Ser21 in GSK-3 α and at Ser9 in GSK-3 β are the major mechanism affecting GSK-3 activity in immune cells (14, 16). NKG2D and 2B4 on rested NKL cells were stimulated, either individually or together, after cross-linking receptors with specific mAbs. Inhibitory phosphorylation of both GSK-3 α and GSK-3 β occurred in NKL cells upon stimulation with either NKG2D or 2B4 alone, and this was further increased in cells stimulated through combination of NKG2D and 2B4 (Fig. 5). Similar results of GSK-3 α/β phosphorylation were observed with primary resting NK cells (Fig. 6). Such enhanced phosphorylation of GSK-3 α/β by combination of NKG2D and 2B4 was similar to the sum of the extent of phosphorylation induced by each receptor, when compared to selective phosphorylation of Akt by NKG2D and synergistic ERK phosphorylation by combination of NKG2D and 2B4 (Fig. 5).

NK cell activation by different receptors is downregulated by the E3 ubiquitin ligase c-Cbl through an inhibition of Vav1-dependent activation signals (7). Thus, activation of NK cells through NKG2D and/or 2B4 is enhanced after depletion of c-Cbl. We found that the inhibitory phosphorylation of GSK-3 α/β by NKG2D, 2B4 or their combination was augmented after small interfering RNA (siRNA)-mediated knockdown of c-Cbl in NKL cells (Fig. 7). This indicates that the extent of GSK-3 phosphorylation correlates with the activation status of NK cells. To confirm this finding, we assessed the change in GSK-3 phosphorylation upon blockade of

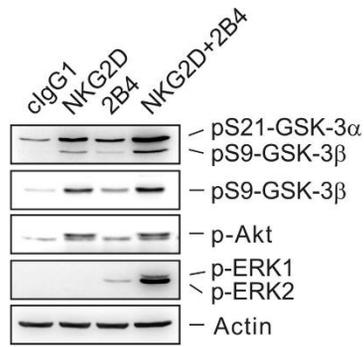


Fig. 5. The phosphorylation of GSK-3 after stimulation of NKG2D and/or 2B4 in NKL cells.

Rested NKL cells were preincubated on ice for 30 min with isotype control mAb (cIgG1) or mAbs specific for NKG2D and/or 2B4, and were then stimulated by receptor crosslinking with secondary goat F(ab')₂ anti-mouse IgG for 2 min at 37 °C. Cell lysates were immunoblotted for pS21/9-GSK-3α/β, pS9-GSK-3β, p-Akt, p-ERK1/2, and actin.

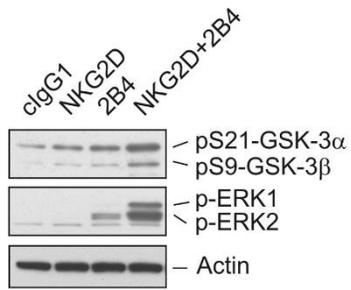


Fig. 6. The phosphorylation of GSK-3 after stimulation of NKG2D and/or 2B4 in primary NK cells.

Primary resting NK cells were preincubated with isotype control mAb or mAbs specific for NKG2D and/or 2B4 and were then stimulated as described in Fig. 5. Cell lysates were immunoblotted for pS21/9-GSK-3 α/β , p-ERK1/2, and actin.

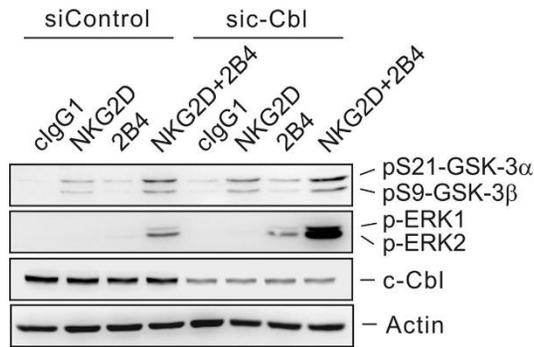


Fig. 7. The phosphorylation of GSK-3 after stimulation of NKG2D and/or 2B4 is increased by knockdown of c-Cbl.

Rested NKL cells transfected with control siRNA or c-Cbl-specific siRNA were preincubated with isotype control mAb or mAbs specific for NKG2D and/or 2B4 and were then stimulated by receptor crosslinking for 2 min. Cell lysates were immunoblotted for pS21/9-GSK-3 α/β , p-ERK1/2, c-Cbl, and actin.

NK cell activation by an inhibitory receptor engagement or Vav1 knockdown. Phosphorylation at Ser21 in GSK-3 α and at Ser9 in GSK-3 β induced by NKG2D, 2B4 or their combination were abrogated by co-crosslinking of CD94-NKG2A inhibitory receptor on NKL cells (Fig. 8). Similar results of GSK-3 α/β phosphorylation were obtained after knockdown of Vav1 (Fig. 9), an essential component in the NKG2D and 2B4 synergy and a primary target protein for inhibitory receptors. Thus, our results indicate that GSK-3 inactivation is induced by activation signals from different activating receptors and correlates with NK cell activation. In support, GSK-3 inhibitors that enhanced NK cell functions augmented the inhibitory phosphorylation of GSK-3 α/β and in turn decreased the phosphorylation of β -catenin, a key substrate of GSK-3 β , induced by NKG2D and 2B4 (Fig. 10).

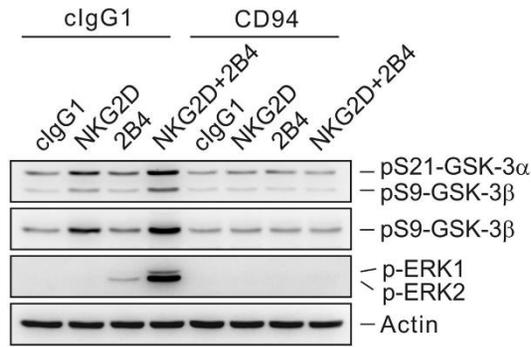


Fig. 8. The phosphorylation of GSK-3 after stimulation of NKG2D and/or 2B4 is inhibited by costimulation of CD94 inhibitory receptor.

Rested NKL cells were preincubated with isotype control mAb or mAbs specific for NKG2D and/or 2B4 combined with or without mAb to CD94 and were then stimulated by receptor crosslinking for 2 min. Cell lysates were immunoblotted for pS21/9-GSK-3 α/β , pS9-GSK-3 β , p-ERK1/2, and actin.

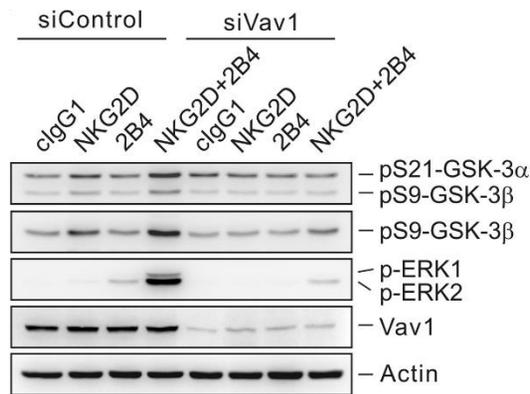


Fig. 9. The phosphorylation of GSK-3 after stimulation of NKG2D and/or 2B4 is inhibited by knockdown of Vav1.

Rested NKL cells transfected with control siRNA or Vav1-specific siRNA were preincubated with the indicated mAbs and were then stimulated by receptor crosslinking for 2 min. Cell lysates were immunoblotted for pS21/9-GSK-3 α/β , pS9-GSK-3 β , p-ERK1/2, Vav1, and actin. The immunoblots are representative of at least three independent experiments.

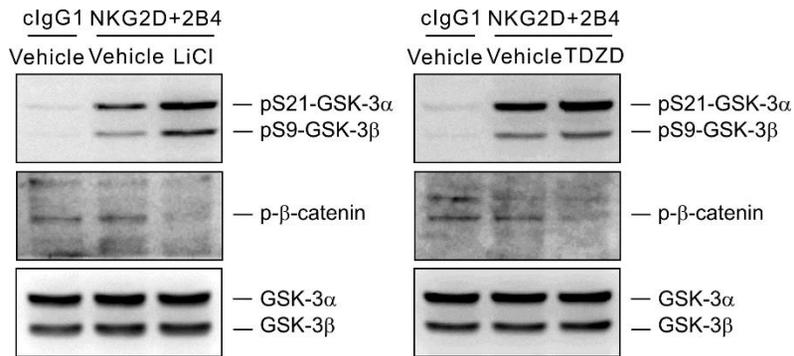


Fig. 10. Small molecule GSK-3 inhibitors cause decrease in β -catenin phosphorylation via an inhibitory phosphorylation of GSK-3 α/β .

Rested NKL cells were preincubated with mAbs specific for NKG2D and 2B4 in the absence or presence of GSK-3 inhibitor LiCl (5 mM) or TDZD-8 (0.5 μ M) for 30 min. After stimulation by receptor crosslinking for 5 min in the presence of inhibitor, cell lysates were immunoblotted for pS21/9-GSK-3 α/β , pS33/37/T41- β -catenin, and GSK-3 α/β . Data are representative of at least two independent experiments.

II-4-3. GSK-3 phosphorylation by PI3K- and MAPK-dependent Pathways

Among the signaling molecules activated by NKG2D and 2B4 synergy and associated with effector function of NK cells are PI3K and MAPK ERK (7, 45-47). These molecules are also implicated in the signaling pathways (e.g. PI3K/Akt, ERK/RSK) related to an inhibitory phosphorylation of GSK-3 at regulatory serine residues (23, 48). Thus, we assessed the sensitivity of GSK-3 phosphorylation to specific inhibitors of such pathways after stimulation of NKL cells through NKG2D and/or 2B4. Phosphorylation at Ser21 in GSK-3 α and at Ser9 in GSK-3 β induced by combination of NKG2D and 2B4 were sensitive to the inhibitors of both PI3K (LY294002) and MEK (PD98059) that diminish phosphorylation of Akt and ERK, respectively (Fig. 11). This result suggests that both PI3K/Akt- and MEK/ERK-dependent pathways are required for the inhibitory phosphorylation of GSK-3. In support of this, GSK-3 phosphorylation induced by NKG2D was largely PI3K/Akt-dependent, whereas GSK-3 phosphorylation by 2B4 was MEK/ERK-dependent (Fig. 11), in agreement with the observation of selective phosphorylation of Akt by NKG2D and of ERK by 2B4. We also found that the combined inhibition of both pathways resulted in an additive effect in decreasing GSK-3 phosphorylation induced by combination of NKG2D and 2B4 (Fig. 12), thus suggesting a coordinated requirement of PI3K/Akt and MEK/ERK pathways for GSK-3 phosphorylation.

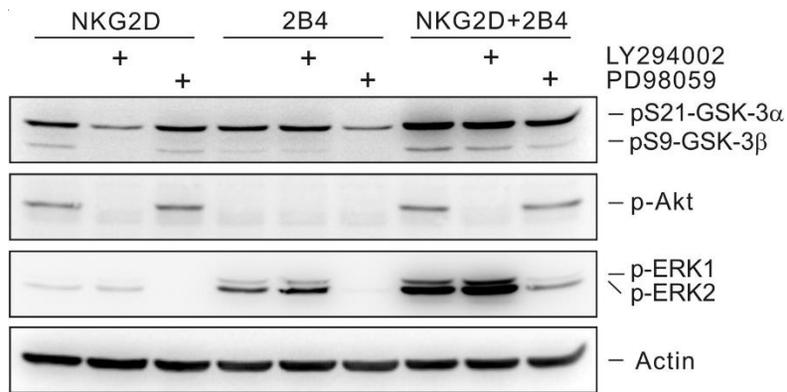


Fig. 11. PI3K and MAPK signaling pathways cooperate to promote GSK-3 phosphorylation.

Rested NKL cells were preincubated with mAbs specific for NKG2D and/or 2B4 in the absence or presence of PI3K inhibitor LY294002 (20 μ M) or MEK inhibitor PD98059 (20 μ M) for 30 min. After stimulation by receptor crosslinking for 2 min in the presence of inhibitor, cell lysates were immunoblotted for pS21/9-GSK-3 α / β , p-Akt, p-ERK1/2, and actin.

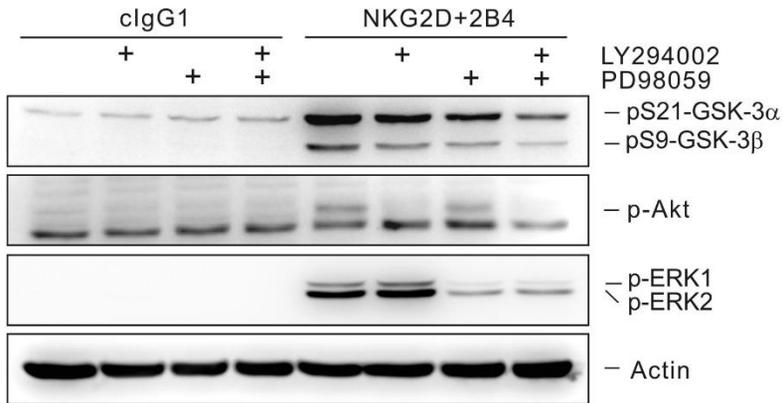


Fig. 12. PI3K and MAPK signaling pathways cooperate to promote GSK-3 phosphorylation in an additive manner.

Rested NKL cells preincubated with isotype control mAb or mAbs specific for NKG2D and 2B4 in the absence or presence of LY294002 (20 μ M) and/or PD98059 (20 μ M) were stimulated by receptor crosslinking for 2 min in the presence of inhibitor. Cell lysates were immunoblotted for pS21/9-GSK-3 α/β , p-Akt, p-ERK1/2, and actin. The immunoblots are representative of at least three independent experiments.

II-4-4. GSK-3 β -dependent regulation of NK cell effector functions

GSK-3 exists as two different isoforms, GSK-3 α and GSK-3 β (22). To determine the individual contribution of these isoforms to the regulation of NK cell functions, we utilized siRNAs specific for each isoform of GSK-3. After transfection of NKL cells with the corresponding siRNAs, a selective and efficient knockdown of GSK-3 α and GSK-3 β was detected in the cells (Fig. 13). As shown in Fig. 14, knockdown of GSK-3 β but not GSK-3 α caused noticeable augmentation in the mRNA expression of IFN- γ and MIP-1 α/β by the engagement of NKG2D, 2B4, or their combination. Similarly, GSK-3 β knockdown significantly enhanced the secretion of IFN- γ via NKG2D and 2B4 combination and of MIP-1 α in response to NKG2D, 2B4, or both (Fig. 15). Moreover, cytotoxicity was significantly enhanced by the knockdown of GSK-3 β but not GSK-3 α , particularly in response to combination of NKG2D and 2B4 (Fig. 16). Thus, these results led us to conclude that effector functions of NK cells, as determined by cytokine/chemokine production and cytotoxicity, triggered by different activating receptors are regulated by GSK-3 β isoform.

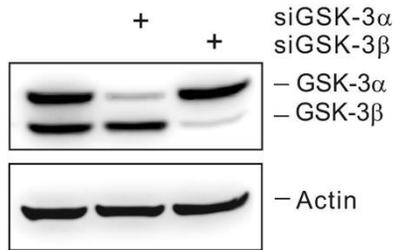


Fig. 13. GSK-3 knockdown with siRNAs specific for each α or β -isoform.

Total lysates of rested NKL cells transfected with control siRNA or siRNAs specific for GSK-3 α and GSK-3 β were immunoblotted for GSK-3 α/β and actin.

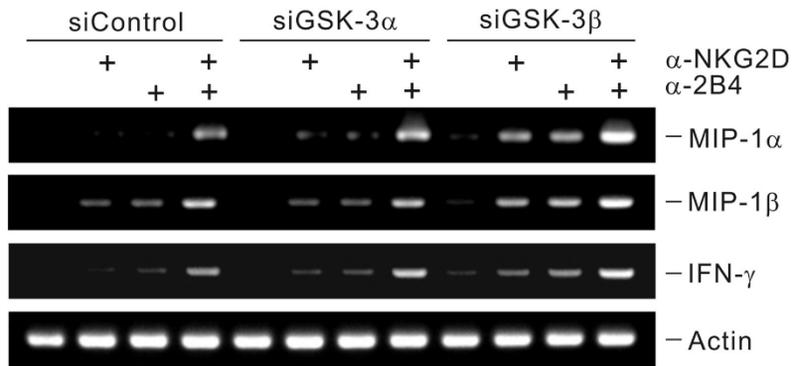


Fig. 14. GSK-3 β isoform selectively regulates NK cell cytokines transcription.

Rested NKL cells transfected with control siRNA, GSK-3 α -specific siRNA, or GSK-3 β -specific siRNA were stimulated with beads coated with isotype control mAb or mAbs specific for NKG2D and/or 2B4. After 3 h incubation, total RNA was isolated and relative mRNA expression of cytokines (IFN- γ , MIP-1 α , MIP-1 β) was assessed by reverse transcription-polymerase chain reaction.

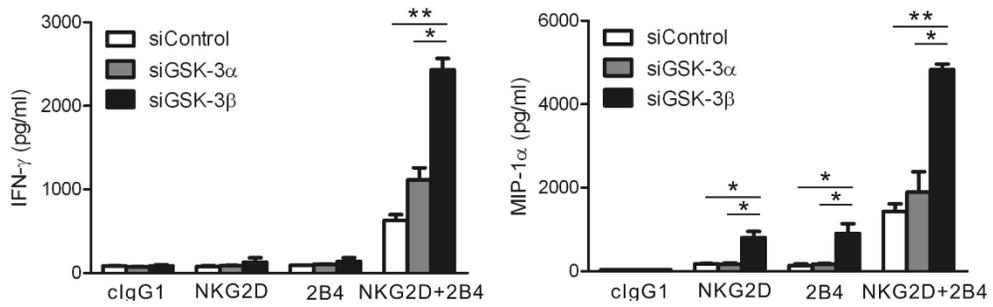


Fig. 15. GSK-3β isoform selectively regulates NK cell cytokines secretion.

Rested NKL cells transfected with control siRNA, GSK-3α-specific siRNA, or GSK-3β-specific siRNA were stimulated as described in Fig. 4B. After 8 h incubation, IFN-γ and MIP-1α in the supernatants were measured by ELISA.

Values represent mean ± SD. *P < 0.05; **P < 0.01

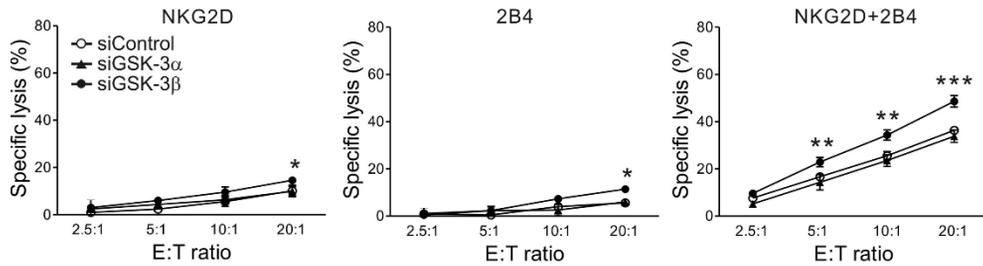


Fig. 16. GSK-3 β isoform selectively regulates NK cell cytotoxicity.

Lysis of P815 cells by rested NKL cells transfected with control siRNA, GSK-3 α -specific siRNA, or GSK-3 β -specific siRNA at the indicated effector to target (E:T) cell ratio. Cytotoxicity against P815 cells preincubated with mAbs specific for NKG2D and/or 2B4 was measured after 2 h with the Europium assay. Error bars represent the SD. Data are representative of at least three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.005.

II-4-5. GSK-3 β regulates calcium-dependent signaling for NK cell Activation

A calcium-mediated signaling pathway that involves PLC- γ and ERK is crucial to the effector functions of NK cells upon target cell recognition (7, 49). Stimulation of NKL cells through combination of NKG2D and 2B4 triggered robust Ca²⁺ mobilization, which was enhanced by the knockdown of GSK-3 β but not GSK-3 α (Fig. 17). Among the calcium-dependent transcription factors that are important for cytokine production such as IFN- γ and are also regulated by GSK-3 β are members of the NFAT family (32, 33, 50, 51). NFAT proteins typically exist as phosphorylated inactive precursors in resting T cells, whereas they are rapidly activated via dephosphorylation upon TCR stimulation. Phosphorylated NFAT proteins have a slower electrophoretic mobility than the dephosphorylated proteins. Stimulation of either NKG2D or 2B4 alone induced marginal or a small increase in the dephosphorylated form of NFAT1, whereas combination of NKG2D and 2B4 led to a marked increase in the dephosphorylated form (Fig. 18A and 18B), which was correlated with PLC- γ 2 phosphorylation and Ca²⁺ influx (Fig. 17 and 18) (7). These changes in the phosphorylation state of NFAT1 were significantly enhanced by knockdown of GSK-3 β (Fig. 18B). Moreover, phosphorylation of ERK, which is a downstream of PLC- γ activation, and of Akt was also significantly augmented by GSK-3 β knockdown (Fig. 18A and 18B). Thus, our results suggest that regulation of NK cell functions by GSK-3 β relies on a mechanism involving modulation of calcium-mediated signaling pathway.

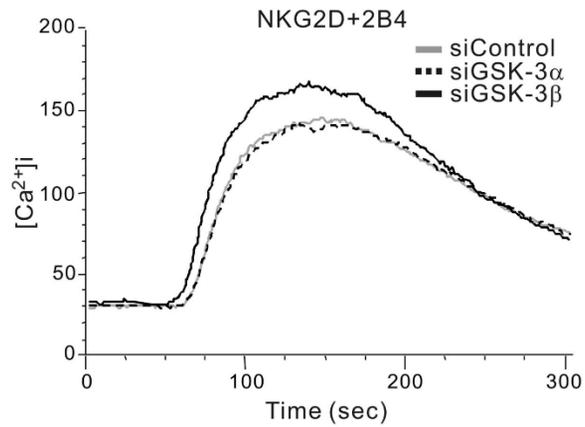
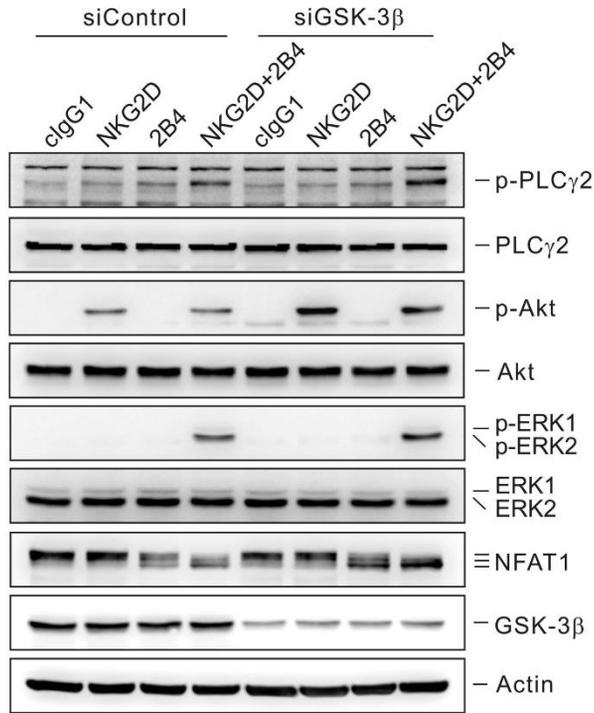


Fig. 17. GSK-3 β knockdown enhances calcium-dependent signaling.

Rested NKL cells transfected with control siRNA, GSK-3 α -specific siRNA, or GSK-3 β -specific siRNA were loaded with Fluo-4 for 30 min at 30 °C and were then stimulated through NKG2D and 2B4 (indicated by the arrow) after the measurement of baseline Ca^{2+} flux for 30 s. Changes in fluorescence by Ca^{2+} mobilization are shown as a function of time.

A



B

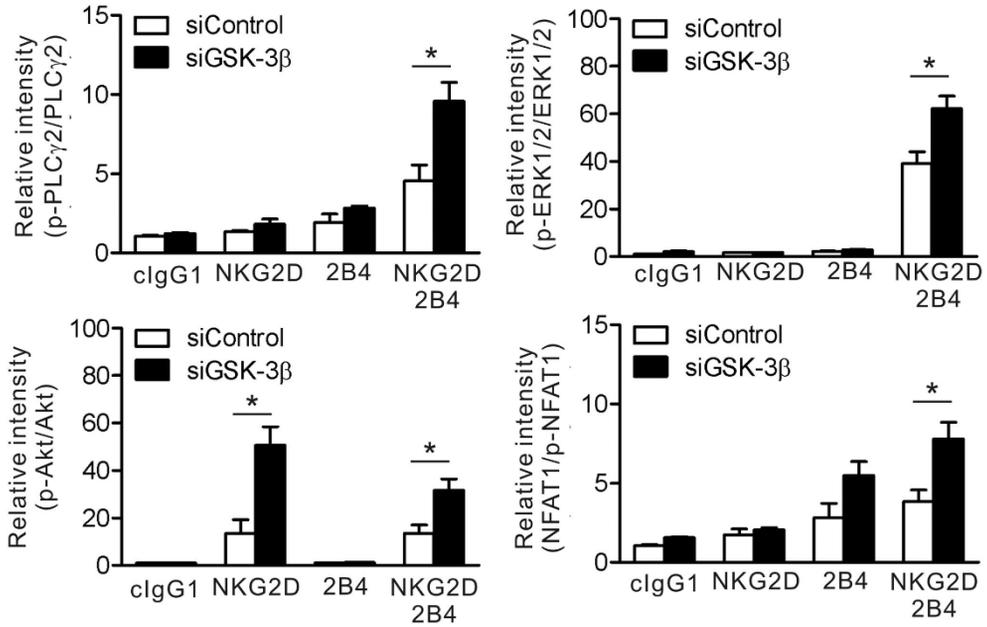


Fig. 18. GSK-3 β knockdown enhances PLC- γ 2 phosphorylation and NFAT1 activity.

(A and B) Rested NKL cells transfected with control siRNA or GSK-3 β -specific siRNA were preincubated with isotype control mAb or mAbs specific for NKG2D and/or 2B4 and were then stimulated by receptor crosslinking for 2 min. Cell lysates were immunoblotted for p-PLC γ 2, PLC γ 2, p-Akt, Akt, p-ERK1/2, ERK1/2, NFAT1, GSK-3 β , and actin (B). Band intensities of phosphorylated proteins relative those of their total forms were quantified with ImageJ software and are presented for each condition (C). Error bars represent the SD. Data are representative of at least three independent experiments. *P < 0.05.

II-4-6. Regulation of NK cell function by GSK-3 β is dependent on its kinase activity

The pharmacological inhibitors of GSK-3 used in our study could reduce the kinase activity of GSK-3. NK cell stimulation through NKG2D and/or 2B4 could also induce GSK-3 β inactivation through inhibitory phosphorylation at Ser9. However, it has been shown that GSK-3 β can regulate inflammatory responses independently of its kinase activity (52). Thus, it prompted us to investigate whether kinase activity of GSK-3 β is directly involved in the regulation of NK cell function. NKL cells were transduced with retroviruses that express wild-type GSK-3 β (WT) or its two mutant forms, constitutively active GSK-3 β (CA; S9A) or kinase dead GSK-3 β (KD; K85A). Due to coexpression of GFP along with the cloned GSK-3 β in the infected cells, cells were sorted for comparable expression of GFP by flow cytometry. The levels of GSK-3 β protein in the transduced NKL cells were similar for wild-type GSK-3 β and its mutants, as measured by flow cytometry (Fig. 19) and immunoblot analysis (Fig. 23A). Overexpression of GSK-3 β KD mutant enhanced, while GSK-3 β CA mutant suppressed the production of IFN- γ and MIP-1 α in response to NKG2D and 2B4 synergy and to NKG2D alone, when compared to overexpression of GSK-3 β wild-type (Fig. 20). Similarly, cytotoxicity was significantly increased by overexpression of GSK-3 β KD mutant and appeared to be decreased by overexpression of GSK-3 β CA mutant under all stimulating conditions (Fig. 21). These results indicate that effector functions of NK cells are regulated by kinase activity of GSK-3 β . We then measured the effects of GSK-3 β mutants on calcium-mediated signaling pathway such as Ca²⁺ influx and a downstream ERK phosphorylation. Compared to overexpression of GSK-3 β wild-type, overexpression

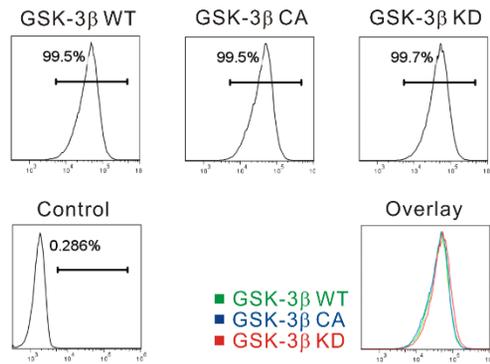


Fig. 19. Establishment of NKL cell line expressing GSK-3 β mutants by retroviral transduction.

NKL cells transduced with retrovirus containing sequences for wild type (WT), constitutively active (CA; S9A mutant), or kinase dead (KD; K85A mutant) form of GSK-3 β were sorted for matched level of GFP expression. The level of GSK-3 β protein as reflected by GFP expression in the transduced cells was measured by flow cytometry.

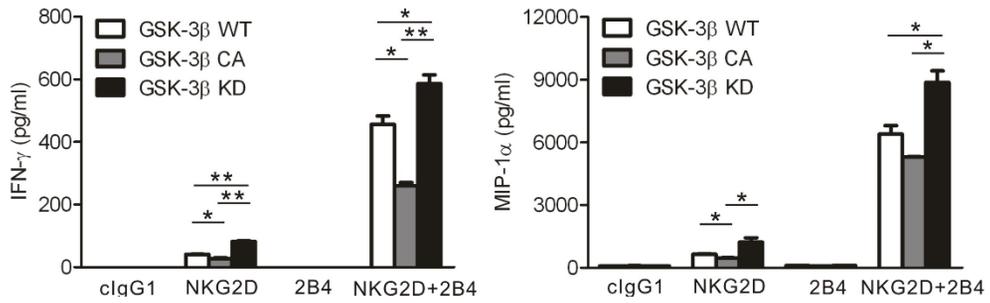


Fig. 20. GSK-3β activity regulates NK cell cytokines secretion.

Rested NKL cells expressing GSK-3β WT, GSK-3β CA or GSK-3β KD were stimulated with beads coated with isotype control mAb or mAbs specific for NKG2D and/or 2B4. After 12 h incubation, IFN-γ and MIP-1α in the supernatants were measured by ELISA. Values represent mean ± SD. *P < 0.05; **P < 0.01.

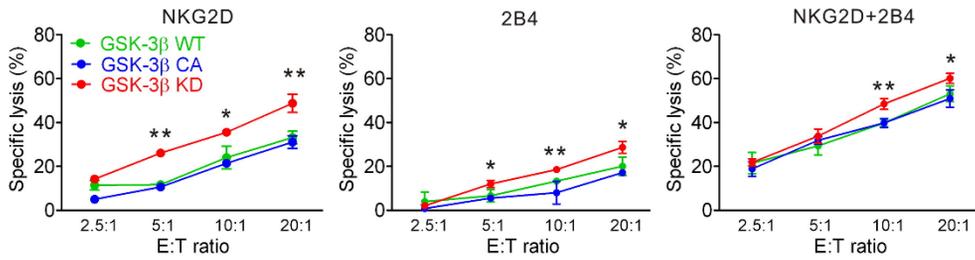


Fig. 21. GSK-3β activity regulates NK cell cytotoxicity.

Lysis of P815 cells by rested NKL cells expressing GSK-3β WT, GSK-3β CA or GSK-3β KD at the indicated effector to target (E:T) cell ratio. Cytotoxicity against P815 cells preincubated with mAbs specific for NKG2D and/or 2B4 was measured after 2 h using the Europium assay. Error bars represent the SD. *P < 0.05; **P < 0.01.

of GSK-3 β KD mutant enhanced, while GSK-3 β CA mutant suppressed, Ca²⁺ mobilization (Fig. 22) and phosphorylation of PLC- γ 2, Akt, and ERK induced by combination of NKG2D and 2B4 (Fig. 23A and 23B). Thus, results suggest that GSK-3 β inactivation is required for efficient NK cell activation through different activating receptors.

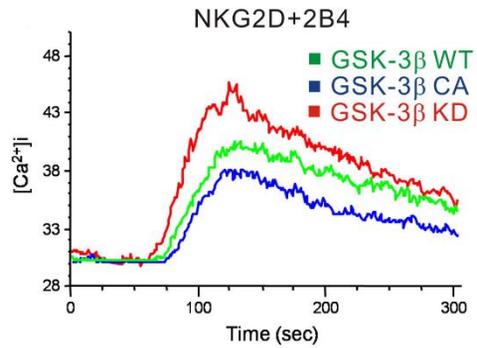


Fig. 22. GSK-3 β activity regulates NK cell calcium signaling.

Calcium mobilization in rested NKL cells expressing GSK-3 β WT, GSK-3 β CA or GSK-3 β KD. The indicated NKL cells were loaded with Asante Calcium Red for 30 min at 30 °C and were then stimulated with NKG2D and 2B4 as described in Fig. 17.

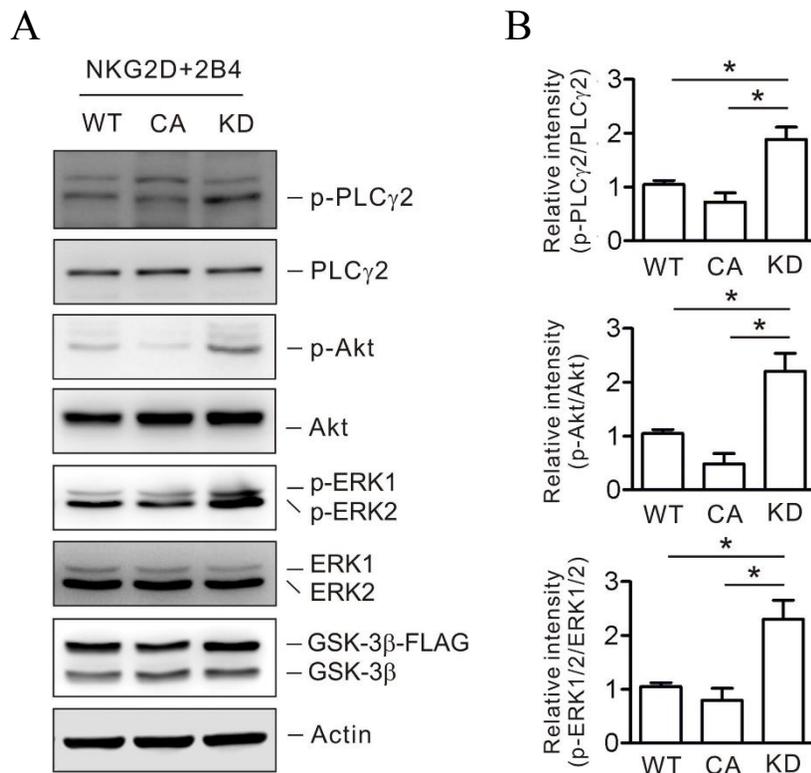


Fig. 23. GSK-3 β activity regulates phosphorylation of PLC- γ 2, Akt, and ERK by combination of NKG2D and 2B4 in NK cell.

(A and B) Rested NKL cells expressing GSK-3 β WT, GSK-3 β CA or GSK-3 β KD were preincubated with mAbs specific for NKG2D and 2B4 and were then stimulated by receptor crosslinking for 2 min. Cell lysates were immunoblotted for p-PLC γ 2, PLC γ 2, p-Akt, Akt, p-ERK1/2, ERK1/2, GSK-3 β , and actin (D). Band intensities of phosphorylated proteins relative those of their total forms were quantified (E). Error bars represent the SD. Data are representative of at least three independent experiments. *P < 0.05.

II-4-7. Role of GSK-3 β in primary NK cell activation

Next, we investigated whether GSK-3 β also regulated NK cell function in primary NK cells and in NK92 cell line. Primary NK cells that were expanded by using cytokines and feeder cells were used as effector cells since cytokine-stimulated NK cells can be triggered by diverse activating receptors on their own (8, 9, 53). First, we performed siRNA-mediated knockdown of GSK-3 β in primary expanded NK cells. After 48 h, we observed a selective and noticeable reduction in the amount of GSK-3 β protein in the cells, as determined by immunoblot analysis (Fig. 24). GSK-3 β knockdown consistently enhanced NK cell expression of IFN- γ upon stimulation with K562 target cells (Fig. 25). Next, we investigated whether the regulation of NK cell function by GSK-3 β also occurred during NK cell activation by different receptors other than NKG2D and 2B4. It has been shown that effector functions of NK cells can be triggered through ITAM-dependent (e.g., CD16, NKp30) and ITAM-independent pathways (e.g., NKG2D, 2B4) (1-3). Stimulation of primary expanded NK cells through CD16 and NKp30 each induced the inhibitory phosphorylation of GSK-3 α/β , as did the stimulation through NKG2D and 2B4 (Fig. 26). After specific knockdown of GSK-3 β in the cells, NK cell expression of IFN- γ was significantly increased upon stimulation with NKG2D and 2B4, NKp30 and CD16 (Fig. 27). Using NK92 cell line, we observed that GSK-3 β knockdown also significantly enhanced the expression of IFN- γ by CD16.NK-92 cells transduced to express CD16 receptor upon stimulation with K562 target cells and CD16 (Fig. 28). Thus, our results suggest that GSK-3 β negatively regulates the activation of NK cells including primary NK cells through both ITAM-coupled and non-ITAM receptors.

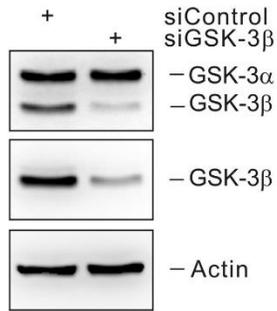


Fig. 24. GSK-3 knockdown with β -isoform-specific siRNAs in primary NK cells.

Total lysates of primary expanded NK cells transfected with control siRNA or GSK-3 β -specific siRNA were immunoblotted for GSK-3 α/β , GSK-3 β , and actin.

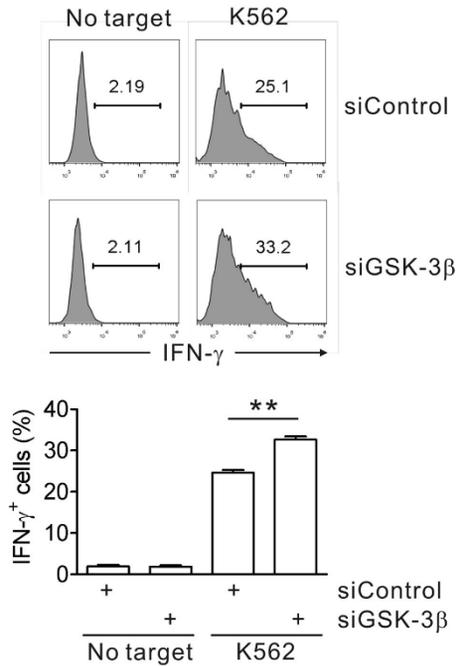


Fig. 25. GSK-3β knockdown enhances IFN-γ expression of primary NK cells induced by K562 target cells.

Primary expanded NK cells transfected with control siRNA or GSK-3β-specific siRNA were mixed with K562 cells for intracellular cytokine assay. After 6 h incubation, cells were stained with fluorochrome-conjugated anti-CD56 mAb for surface staining. Cytokine production by NK cells was measured in CD56+ cells by flow cytometry after intracellular staining of IFN-γ. Representative FACS profiles (top) and graphs of statistical bar charts (bottom) showing expression of IFN-γ by NK cells. Values represent mean ± SD. **P < 0.01

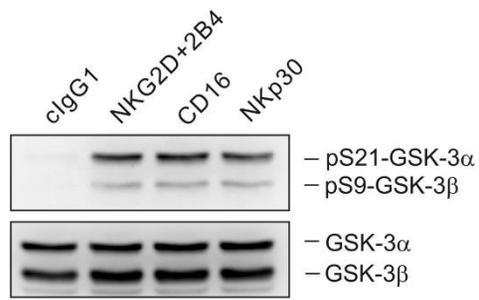


Fig. 26. The phosphorylation of GSK-3 after stimulation of both ITAM- and non-ITAM-dependent receptors in primary NK cells.

Primary expanded NK cells were preincubated with isotype control mAb or mAbs specific for NKG2D and 2B4, CD16, or NKp30 and were then stimulated by receptor crosslinking for 2 min. Cell lysates were immunoblotted for pS21/9-GSK-3 α/β , and GSK-3 α/β .

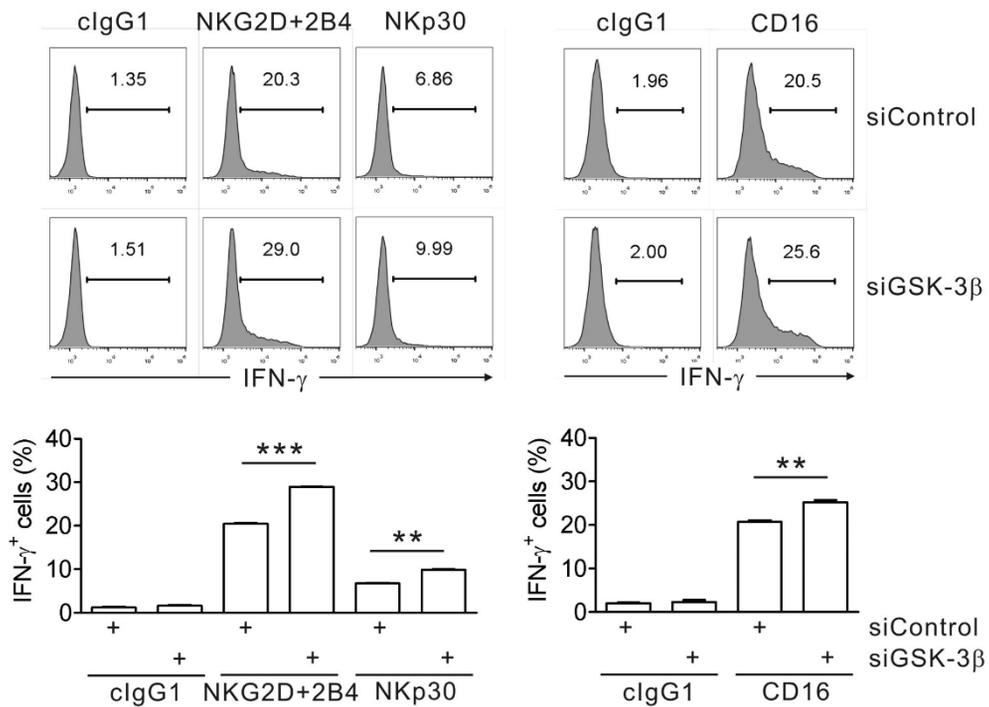


Fig. 27. GSK-3 β knockdown enhances IFN- γ expression of primary NK cells induced by both ITAM- and non-ITAM-dependent receptors.

Primary expanded NK cells transfected with control siRNA or GSK-3 β -specific siRNA were mixed with P815 cells preincubated with isotype control mAb or mAbs specific for NKG2D and 2B4, CD16, or NKp30 for intracellular cytokine assay. After 6 h incubation, cells were analyzed by flow cytometry as described in Fig. 25 for IFN- γ production by NK cells. Representative FACS profiles (top) and graphs of statistical bar charts (bottom) showing expression of IFN- γ by NK cells. Error bars represent the SD. Data are representative of at least three independent experiments. **P < 0.01; ***P < 0.005.

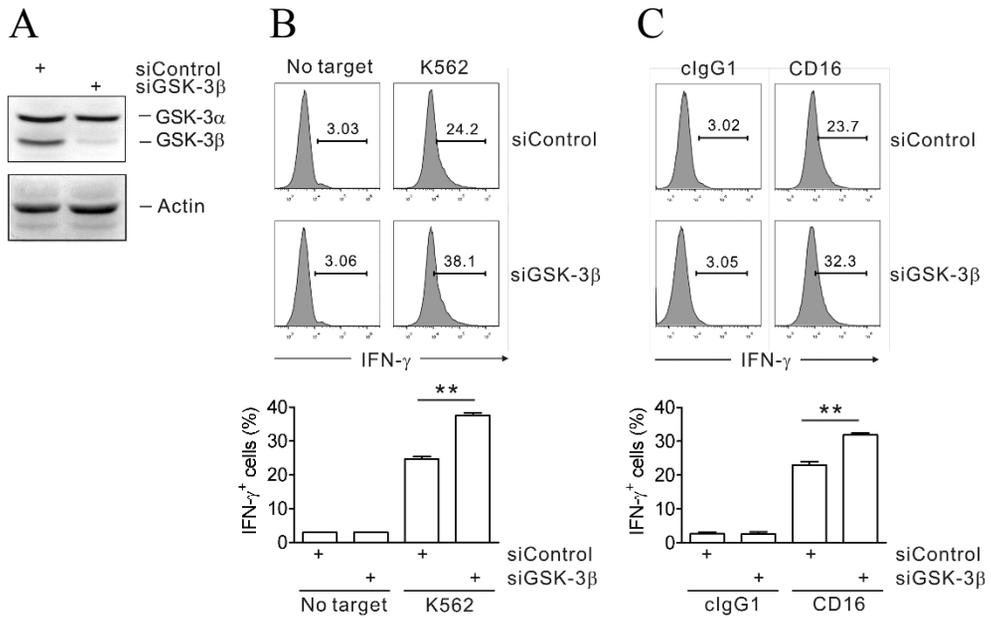


Fig. 28. Enhanced IFN- γ production by CD16.NK92 cells after GSK-3 β knockdown.

(A) Total lysates of CD16.NK92 cells transfected with control siRNA or GSK-3 β -specific siRNA were immunoblotted for GSK-3 α/β and actin.

(B and C) CD16.NK92 cells transfected with control siRNA or GSK-3 β -specific siRNA were mixed with (B) K562 cells or (C) P815 cells preincubated with isotype control mAb or mAb to CD16 for intracellular cytokine assay. After 6 hr incubation, cells were stained with fluorochrome-conjugated anti-CD56 mAb for surface staining. Cytokine production by NK cells was measured in CD56 $^+$ cells by flow cytometry after intracellular staining of IFN- γ . Representative FACS profiles (top) and graphs of statistical bar charts (bottom) showing expression of IFN- γ by CD16.NK92 cells. Values represent mean \pm SD. Data are representative of at least two independent experiments. **P < 0.01.

II-5. Discussion

In this study, we present evidence to support that GSK-3, specifically GSK-3 β , is a crucial signaling molecule for the regulation of NK cell function through multiple activating receptors. Using a model of NK cell activation through combination of NKG2D and 2B4 that couple to different signaling adaptors, we found that an inhibitory phosphorylation of GSK-3 β at Ser9 was induced in common by the engagement of NKG2D, 2B4, or their combination. Of interest, such a mechanism of GSK-3 β inactivation was required for the receptors to augment NK cell functions such as cytokine production and cytotoxicity. Moreover, activating receptors associated with ITAM-containing adaptor proteins such as CD16 and NKp30 also induced the inactivation of GSK-3 β , the depletion of which potentiated the NK cell response through CD16 and NKp30. Thus, our results suggest that GSK-3 β negatively regulates NK cell responses induced by diverse activating receptors and that the inhibition of GSK-3 β is required to mount enhanced NK cell responses.

Although initially discovered as a metabolic kinase, GSK-3 is now appreciated as a critical regulator of inflammatory responses in both innate and adaptive immune cells (17). However, there are conflicting reports regarding the role of GSK-3 in the regulation of inflammatory cytokine production depending on the types of stimuli and immune cells. GSK-3 β promotes inflammatory cytokine production by monocytes following TLR stimulation (28). Likewise, inhibition of GSK-3 by pharmacological agents or molecular manipulation (e.g., knockdown of GSK-3 β) reduces the production of various inflammatory cytokines triggered by diverse stimuli in dendritic cells and mast cells (29, 30). In contrast, in Ag-specific

stimulation of T cells, GSK-3 inactivation increases IL-2 production and cell proliferation, thus partially substituting for CD28 costimulation (31-33). In this study, we demonstrated that the inhibition of GSK-3 β consistently potentiated NK cell responses, such as cytokine production and cytotoxicity, by NK cells stimulated with diverse activating receptors coupled to both ITAM- and non-ITAM-containing adaptors. In this regard, the regulation of inflammatory cytokine production by GSK-3 appears to be context-dependent according to the types of stimuli and immune cells, thus implying that GSK-3 may have the potential to regulate certain signaling pathways for cytokine production in both positive and negative ways.

GSK-3 consists of two different isoforms, GSK-3 α and GSK-3 β , both of which are implicated in a diverse range of cellular functions (17, 54-56). Although these isoforms appear to be redundant in certain cellular functions such as the regulation of Wnt/ β -catenin signaling (24), there are increasing reports regarding the isoform-specific functions of GSK-3. In this regard, we attempted to investigate whether GSK-3 α and GSK-3 β regulate NK cell functions differently. In a previous study, effector functions of NK cells against target cells were increased by treatment with a GSK-3 inhibitor (34). However, this study was based solely on the pharmacological inhibition of GSK-3 with a single agent, TDZD-8. Although TDZD-8 is originally described as a GSK-3 β inhibitor, a recent study reported its unique ability to induce selective death of leukemia stem cells that appears to be independent of GSK-3 β (57). In addition, instead of GSK-3 phosphorylation at regulatory serine residues, they studied the overall tyrosine phosphorylation of GSK-3 upon 2B4 stimulation. Finally, in the presence of TDZD-8, NK cells were stimulated with target cells combined with 2B4 but not with 2B4 alone, therefore the

effect of GSK-3 on NK cell functions in the context of defined receptor stimulation remains unknown. In this regard, to assess the role of each GSK-3 isoform in NK cell functions, we used isoform-specific siRNAs in addition to three different classes of GSK-3 inhibitors. NK cells were also stimulated with diverse activating receptors, individually or together, in addition to sensitive target cells. We showed that NK cell functions were selectively regulated by GSK-3 β rather than GSK-3 α although both isoforms underwent inhibitory phosphorylation upon stimulation of NK cells. This observation was further supported by the finding with GSK-3 β mutants showing that the regulation of NK cell functions by GSK-3 β relied on its kinase activity.

It has been shown that the phosphorylation of GSK-3 β at Ser9 is the predominant mechanism that results in GSK-3 β inactivation in various types of immune cells (17, 19). Among several signaling pathways implicated in the induction of inhibitory phosphorylation of GSK-3 β (23), we found that the PI3K/Akt and MEK/ERK pathways, either individually or together, were involved in such a phosphorylation in NK cells. Stimulation of either NKG2D or 2B4 alone induced GSK-3 β phosphorylation at Ser9 largely through the PI3K/Akt- or MEK/ERK-dependent pathway, respectively. In comparison, combination of NKG2D and 2B4 triggered synergistic ERK phosphorylation and the NKG2D-dependent Akt phosphorylation, both of which mediated cooperative phosphorylation of GSK-3 β at Ser9. In addition, ERK associates with and phosphorylates GSK-3 β at Thr43, which primes GSK-3 β for its subsequent phosphorylation at Ser9 by RSK, a kinase downstream of ERK activation, thus underscoring the importance of ERK pathway in GSK-3 β inactivation (58). Of interest, a previous study demonstrated that engagement of diverse activating receptors including both ITAM-coupled (e.g.,

NKp30, NKp46) and non-ITAM receptors (e.g., NKG2D, 2B4) commonly led to ERK phosphorylation, although they use distinct proximal signaling modules (59). Moreover, NKG2D and ITAM-bearing receptors such as CD16 are known to trigger PI3K/Akt pathway (1, 10, 60-62). Therefore, the activation of PI3K/Akt and/or MEK/ERK pathway by multiple, if not all, activating receptors may explain why GSK-3 β is broadly implicated in the multiple activation pathways for NK cell activation. In support of this, we observed the inhibitory phosphorylation of GSK-3 β upon stimulation of NK cells through ITAM-coupled CD16 and NKp30. The extent of inhibitory phosphorylation of GSK-3 β correlated with the degree of NK cell activation: such a phosphorylation was induced by either NKG2D or 2B4 alone and was further enhanced by their synergistic combination. Given the augmentation of NK cell function by GSK-3 β inactivation, GSK-3 β may therefore function as a checkpoint protein for NK cell activation.

A mechanistic study indicated that enhanced NK cell functions by GSK-3 β inhibition could be attributed to an increase in calcium-mediated signaling pathway that has a crucial role in NK cell responses upon target cell recognition (49). This pathway that includes PLC- γ , Ca²⁺ mobilization, and ERK is commonly triggered by multiple activating receptors, alone or in combination (1, 59). Calcium signaling has also been shown to activate NFAT transcription factor via dephosphorylation of NFAT, which is critical to the transcription of several inflammatory cytokines including IFN- γ in TCR-stimulated CD4⁺ T cells (51) and CD16-stimulated NK cells (50). Elevated Ca²⁺ levels activate the phosphatase calcineurin, which dephosphorylates NFAT proteins and allows its subsequent nuclear import for target gene transcription (63, 64). Accumulating evidences suggest that GSK-3 β functions

as a NFAT kinase and thereby inhibits NFAT activation through direct phosphorylation of NFAT at conserved serine residues necessary for nuclear export (33, 65). In this study, we observed a robust Ca^{2+} mobilization and an apparent NFAT1 activation (dephosphorylation) in response to combination of NKG2D and 2B4, both of which were further enhanced by GSK-3 β inhibition. Thus, such an enhancement of NFAT1 activation by GSK-3 β inhibition might be accounted for by a dual mechanism: reduced nuclear export of NFAT1 and/or its increased nuclear import. Of interest, we observed that GSK-3 β inhibition in NK cells led to an increase in activating phosphorylation of Akt and ERK as well as Ca^{2+} mobilization. Given a crucial role of Akt and ERK pathways in mediating inhibitory phosphorylation of GSK-3 β , our results suggest that there may be a feedback regulation between Akt and GSK-3 β or between ERK and GSK-3 β . In support of this, a previous study reported that pharmacological inhibition of GSK-3 β resulted in increased phosphorylation of Akt through inactivation of phosphatase and tensin homolog (PTEN) during receptor activator of nuclear factor κ B ligand (RANKL) signaling (66). Moreover, inhibition of GSK-3 via inhibitors and siRNAs was shown to increase the phosphorylation of ERK through PKC- δ in human colon cancer cells (67). However, further study will be required to elucidate the mechanism(s) by which GSK-3 β acts to regulate the activation of Akt and ERK in NK cells.

Animal and clinical studies have shown that the lack of NK cell effector functions is a crucial risk factor associated with the development of various types of cancer (68-70). In addition, the degree of NK cell dysfunction correlates with the prognosis of cancer patients (70-72). This correlation has encouraged continuous interest and further studies intended to develop strategies to improve NK cell

responsiveness against cancer cells. In this regard, targeted modulation of GSK-3, specifically GSK-3 β , in NK cells may be a new strategy to harness NK cell effector function against cancer cells, given that GSK-3 β inactivation including pharmacological inhibitors potentiated NK cell activation induced by diverse activating receptors. Moreover, a recent study showed that GSK-3 inhibition also promoted the upregulation of MICA, a ligand for NKG2D, on multiple myeloma cells and thereby increased their susceptibility to NK cell-mediated cytotoxicity (73). Thus, such combined benefits of GSK-3 inhibition may support the notion of GSK-3 inhibition as a potential therapeutic strategy against certain type of cancer cells in association with NK cells.

II-6. References

1. Long, E.O., Kim, H.S., Liu, D., Peterson, M.E. & Rajagopalan, S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu. Rev. Immunol.* **31**, 227-258 (2013).
2. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat. Immunol.* **9**, 503-510 (2008).
3. Lanier, L.L. NK cell recognition. *Annu. Rev. Immunol.* **23**, 225–274 (2005).
4. Moretta, L. & Moretta, A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *EMBO J.* **23**, 255-259 (2004).
5. Bryceson, Y.T., March, M.E., Ljunggren, H.G. & Long, E.O. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107**, 159-166 (2006).
6. Bryceson, Y.T., Ljunggren, H.G. & Long, E.O. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. *Blood* **114**, 657-666 (2009).
7. Kim, H.S., Das, A., Gross, C.C., Bryceson, Y.T. & Long, E.O. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity* **32**, 175-186 (2010).
8. Cooper, M.A. *et al.* Cytokine-induced memory-like natural killer cells. *Proc. Natl. Acad. Sci. USA* **106**, 1915-1919 (2009).

9. Romme, R. *et al.* Cytokine activation induces human memory-like NK cells. *Blood* **120**, 4751-4760 (2012).
10. Lanier, L.L. Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* **9**, 495-502 (2008).
11. Vivier, E., Nunes, J.A. & Vely, F. Natural killer cell signaling pathways. *Science* **306**, 1517-1519 (2004).
12. Cella, M. *et al.* Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. *J. Exp. Med.* **200**, 817-823 (2004).
13. Tassi, I. *et al.* Phospholipase C- γ 2 is a critical signaling mediator for murine NK cell activating receptors. *J. Immunol.* **175**, 749-754 (2005).
14. Caraux, A. *et al.* Phospholipase C- γ 2 is essential for NK cell cytotoxicity and innate immunity to malignant and virally infected cells. *Blood* **107**, 994-1002 (2006).
15. Ombrello, E.F. *et al.* Cold urticaria, immunodeficiency, and autoimmunity related to *PLCG2* deletions. *N. Engl. J. Med.* **366**, 330-338 (2012).
16. Upshaw, J.L., Schoon, R.A., Dick, C.J., Billadeau, D.D. & Leibson, P.J. The isoforms of phospholipase C- γ are differentially used by distinct human NK activating receptors. *J. Immunol.* **175**, 213-218 (2005).
17. Beurel, E., Michalek, S.M. & Jope, R.S. Innate and adaptive immune responses regulated by glycogen synthase kinase-3 (GSK3). *Trends Immunol.* **31**, 24-31 (2010).

18. Jope, R.S. & Johnson, G.V. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem. Sci.* **29**, 95-102 (2004).
19. Wang, H., Brown, J. & Martin, M. Glycogen synthase kinase 3: A point of convergence for the host inflammatory response. *Cytokine* **53**, 130-140 (2011).
20. Cohen, P. & Goedert, M. GSK3 inhibitors: development and therapeutic potential. *Nat. Rev. Drug Discov.* **3**, 479-487 (2004).
21. Woodgett, J.R. Physiological roles of glycogen synthase kinase-3: Potential as a therapeutic target for diabetes and other disorders. *Curr. Drug Targets Immune Endocr. Metabol. Disord.* **3**, 281-290 (2003).
22. Woodgett, J.R. Molecular cloning expression of glycogen synthase kinase-3/Factor A. *EMBO J.* **9** 2431-2438 (1990).
23. Cohen, P. & Frame, S. The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* **2**, 769-776 (2001).
24. Doble, B.W., Patel, S., Wood, G.A., Kockeritz, L.K. & Woodgett, J.R. Functional redundancy of GSK-3 α and GSK-3 β in Wnt/ β -Catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev. Cell* **12**, 957-971 (2007).
25. Hoeflich, K.P. *et al.* Requirement for glycogen synthase kinase-3 β in cell survival and NF- κ B activation. *Nature* **406**, 86-90 (2000).
26. Kerkela, R. *et al.* Deletion of GSK-3 β in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation. *J. Clin. Invest.* **118**, 3609-3618 (2008).

27. MacAulay, K. *et al.* Glycogen synthase kinase 3 α -specific regulation of murine hepatic glycogen metabolism. *Cell Metab.* **6**, 329-337 (2007).
28. Martin, M., Rehani, K., Jope, R.S. & Michalek, S.M. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* **6**, 777-784 (2005).
29. Rodionova, E. *et al.* GSK-3 mediates differentiation and activation of proinflammatory dendritic cells. *Blood* **109**, 1584-1592 (2007).
30. Radinger, M., Kuehn, H.S., Kim, M.S., Metcalfe, D.D. & Gilfillan, A.M. Glycogen synthase kinase 3 β activation is a prerequisite signal for cytokine production and chemotaxis in human mast cells. *J. Immunol.* **184**, 564-572 (2010).
31. Garcia, C.A. *et al.* Antigenic experience dictates functional role of glycogen synthase kinase-3 in human CD4⁺ T cell responses. *J. Immunol.* **181**, 8363-8371 (2008).
32. Diehn, M. *et al.* Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proc. Natl. Acad. Sci. USA* **99**, 11796-11801 (2002).
33. Ohteki, T. *et al.* Negative regulation of T cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. *J. Exp. Med.* **192**, 99-104 (2000).
34. Aoukaty, A. & Tan, R. Role for glycogen synthase kinase-3 in NK cell cytotoxicity and X-linked lymphoproliferative disease. *J. Immunol.* **174**, 4551-

4558 (2005).

35. Tassi, I., Klesney-Tait, J., Colonna, M. Dissecting natural killer cell activation pathways through analysis of genetic mutations in human and mouse. *Immunol. Rev.* **214**, 92-105 (2006).
36. Kim, H.S. *et al.* Attenuation of natural killer cell functions by capsaicin through a direct and TRPV1 independent mechanism. *Carcinogenesis* **35**, 1652-1660 (2014).
37. Binyamin, L. *et al.* Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy. *J. Immunol.* **180**, 6392-6401 (2008).
38. Anikeeva, N. *et al.* Integrin receptors on tumor cells facilitate NK-cell-mediated antibody-dependent cytotoxicity. *Eur. J. Immunol.* **44**, 2331-2339 (2014).
39. Fujisaki, H. *et al.* Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res.* **69**, 4010-4017 (2009).
40. Bryceson, Y.T., March, M.E., Barber, D.F., Ljunggren, H.G. & Long, E.O. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J. Exp. Med.* **202**, 1001-1012 (2005).
41. Fauriat, C., Long, E.O., Ljunggren, H.G. & Bryceson, Y.T. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* **115**, 2167-2176 (2010).
42. Bryceson, Y.T. *et al.* Functional analysis of human NK cells by flow cytometry. *Methods Mol. Biol.* **612**, 335-352 (2010).

43. Kim, H.S. & Long, E.O. Complementary phosphorylation sites in the adaptor protein SLP-76 promotes synergistic activation of natural killer cells. *Sci. Signal.* **5**, ra49 (2012).
44. Eldar-Finkelman, H. & Martinez, A. GSK-3 inhibitors: Preclinical and clinical focus on CNS. *Front. Mol. Neurosci.* **4**, 32 (2011).
45. Tassi, I. *et al.* p110 γ and p110 δ phosphoinositide 3-kinase signaling pathways synergize to control development and functions of murine NK cells. *Immunity* **27**, 214-227 (2007).
46. Trotta, R. *et al.* Dependence of both spontaneous and antibody-dependent, granule exocytosis-mediated NK cell cytotoxicity on extracellular signal-regulated kinases. *J. Immunol.* **161**, 6648-6656 (1998).
47. Wei, S. *et al.* Control of lytic function by mitogen-activated protein kinase/extracellular regulatory kinase 2 (ERK2) in a human natural killer cell line: Identification of perforin and granzyme B mobilization by functional ERK2. *J. Exp. Med.* **187**, 1753-1765 (1998).
48. Stambolic, V. & Woodgett, J.R. Mitogen inactivation of glycogen synthase kinase-3 β in intact cells via serine 9 phosphorylation. *Biochem. J.* **303**, 701-704 (1994).
49. Maul-Pavicic, A. *et al.* ORAI1-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis. *Proc. Natl. Acad. Sci. USA* **108**, 3324-3329 (2011).
50. Aramburu, J., Azzoni, L., Rao, A. & Perussia, B. Activation and expression of

the nuclear factors of activated T cells, NFATp and NFATc, in human natural killer cells: regulation upon CD16 ligand binding. *J. Exp. Med.* **182**, 801-810 (1995).

51. Kiani, A. *et al.* Regulation of interferon- γ gene expression by nuclear factor of activated T cells. *Blood* **98**, 1480-1488 (2001).
52. Lei, C.Q. *et al.* Glycogen synthase kinase 3 β regulates IRF3 transcription factor-mediated antiviral response via activation of the kinase TBK1. *Immunity* **33**, 878-889 (2010).
53. Itoh, S. *et al.* GSK-3 α and GSK-3 β proteins are involved in early stages of chondrocyte differentiation with functional redundancy through RelA protein phosphorylation. *J. Biol. Chem.* **287**, 29227-29236 (2012).
54. Doble, B.W. & Woodgett, J.R. GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell Sci.* **116**, 1175-1186 (2003).
55. Force, T. & Woodgett, J.R. Unique and overlapping functions of GSK-3 isoforms in cell differentiation and proliferation and cardiovascular development. *J. Biol. Chem.* **284**, 9643-9647 (2009).
56. Frame, S. & Cohen, P. GSK3 takes centre stage more than 20 years after its discovery. *Biochem. J.* **359**, 1-16 (2001).
57. Guzman, M.L. *et al.* Rapid selective death of leukemia stem and progenitor cells induced by the compound 4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione (TDZD-8). *Blood* **110**, 4436-4444 (2007).
58. Ding, Q. *et al.* Erk associates with and primes GSK-3 β for its inactivation

- resulting in upregulation of β -catenin. *Mol. Cell* **19**, 159-170 (2005).
59. Chen, X., Trivedi, P.P., Ge, B., Krzewski, K. & Strominger, J.L. Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. *Proc. Natl. Acad. Sci. USA* **104**, 6329-6334 (2007).
60. Billadeau, D.D., Upshaw, J.L., Schoon, R.A., Dick, C.J. & Leibson, P.J. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat. Immunol.* **4**, 557-564 (2003).
61. Bonnema, J.D., Karnits, L.M., Schoon, R.A., Abraham, R.T. & Leibson, P.J. Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells is associated with protein kinase C-independent granule release and cell-mediated cytotoxicity. *J. Exp. Med.* **180**, 1427-1435 (1994).
62. Jiang, K. *et al.* Syk regulation of phosphoinositide 3-kinase-dependent NK cell function. *J. Immunol.* **168**, 3155-3164 (2002).
63. Crabtree, G.R. *et al.* Generic signals and specific outcomes: signaling through Ca^{2+} , calcineurin, and NF-AT. *Cell* **96**, 611-614 (1999).
64. Shibasaki, F., Price, E.R., Milan, D. & McKeon, F. Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature* **382**, 370-373 (1996).
65. Beals, C.R., Sheridan, C.M., Turck, C.W., Gardner, P. & Crabtree, G.R. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* **275**, 1930-1934 (1997).

66. Jang, H.D., Noh, J.Y., Shin, J.H., Lin, J.J. & Lee, S.Y. PTEN regulation by the Akt/GSK-3 β axis during RANKL signaling. *Bone* **55**, 126-131 (2013).
67. Wang, Q., Zhou, Y., Wang, X. & Evers, B.M. Glycogen synthase kinase-3 is a negative regulator of extracellular signal-regulated kinase. *Oncogene* **25**, 43-50 (2006).
68. Talmadge, J.E., Meyers, K.M., Prieur, D.J. & Starkey, J.R. Role of NK cells in tumor growth and metastasis in *beige* mice. *Nature* **284**, 622-624 (1980).
69. Oka, M., Mitsunaga, H., Hazama, S., Yoshino, S. & Suzuki, T. Natural killer activity and serum immunosuppressive acidic protein levels in esophageal and gastric cancers. *Surg. Today* **23**, 669-674 (1993).
70. Imai, K., Matsuyama, S., Miyake, S., Suga, K. & Nakachi, K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* **356**, 1795-1799 (2000).
71. Takeuchi, H. *et al.* Prognostic significance of natural killer cell activity in patients with gastric carcinoma: a multivariate analysis. *Am. J. Gastroenterol.* **96**, 574-578 (2001).
72. Mamessier, E. *et al.* Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *J. Clin. Invest.* **121**, 3609-3622 (2011).
73. Fionda, C. *et al.* Inhibition of glycogen synthase kinase-3 increases NKG2D ligand MICA expression and sensitivity to NK cell-mediated cytotoxicity in multiple myeloma cells: role of STAT3. *J. Immunol.* **190**, 6662-6672 (2013).

Chapter III.

**Stepwise phosphorylation of p65
promotes NF- κ B activation and NK
cell responses during target cell
recognition**

Published in

Nature Communications 7 (2016) 11686

III-1. Abstract

NF- κ B is a key transcription factor that dictates the outcome of diverse immune responses. How NF- κ B is regulated by multiple activating receptors that are engaged during natural killer (NK)-target cell contact remains undefined. Here we show that sole engagement of NKG2D, 2B4, or DNAM-1 is insufficient for NF- κ B activation. Rather, cooperation between these receptors is required at the level of Vav1 for synergistic NF- κ B activation. Vav1-dependent synergistic signaling requires a separate PI3K-Akt signal, primarily mediated by NKG2D or DNAM-1, for optimal p65 phosphorylation and NF- κ B activation. Vav1 controls downstream p65 phosphorylation and NF- κ B activation. Synergistic signaling is defective in X-linked lymphoproliferative disease (XLP1) NK cells entailing 2B4 dysfunction and required for p65 phosphorylation by PI3K-Akt signal, suggesting stepwise signaling checkpoint for NF- κ B activation. Thus, our study provides a framework explaining how signals from different activating receptors are coordinated to determine specificity and magnitude of NF- κ B activation and NK cell responses.

III-2. Introduction

Natural killer (NK) cells serve pivotal roles in the early defense against transformed and virus-infected cells and also help shape adaptive immune responses by regulating antigen-presenting cells and T-cell responses (1, 2). These effector functions involve the secretion of cytokines such as interferon- γ (IFN- γ) and TNF- α and the contact-dependent cytolysis of target cells (3). NK cells can mount selective responses against diseased cells via integration of signals delivered by an array of germ line-encoded receptors (1). To avoid inappropriate NK cell reactivity towards healthy cells, signals from multiple activating receptors are kept in check by inhibitory receptors such as killer cell Ig-like receptors (KIRs) and CD94-NKG2A heterodimer specific for MHC class I molecules on target cells. Even in the absence of such inhibition, engagement of a single activating receptor is generally insufficient to activate resting human NK cells due to a cell-intrinsic inhibition mechanism (4). Efficient activation of resting NK cells requires combined stimulation by particular pairs of coactivation receptors, which function in combination (hereafter referred to as “synergistic” signaling). This differs from the activation of cytokine-stimulated NK cells, which no longer require coactivation (5, 6).

Receptor combinations that function synergistically include 2B4 (CD244) paired with NKG2D (CD314) or DNAM-1 (CD226), each with its unique signaling properties. 2B4 carries an ITSM motif in its cytoplasmic tail and transmits activation signals through recruitment of the small adaptor SAP and SAP-associated tyrosine

kinase Fyn (7, 8). 2B4 signaling leads to Vav1, p38 MAPK, Erk, and PLC- γ 2 activation (9). Notably, in NK cells from patients with the inherited immunodeficiency X-linked lymphoproliferative disease (XLP1), which lack functional SAP expression, 2B4 fails to activate and may instead deliver inhibitory signals (10). NKG2D associates with the adaptor DAP10, which carries a YINM motif and signals through recruitment of phosphatidylinositol-3-kinase (PI3K) or Grb2-Vav1 complex (11). NKG2D signaling involves Akt and MAPK Erk and Jnk. DNAM-1 signaling in NK cells remains unclear. DNAM-1 is associated with Fyn and phosphorylated by protein kinase C (12), which is required for optimal differentiation of memory NK cells during cytomegalovirus infection (13).

NK cell activation through receptors for ligands present on target cells can stimulate early cytokine and chemokine production, as well as target cell killing. A recent study on distinct NK subsets revealed CD56^{dim} NK cells, which are regarded as being specialized in cytotoxicity, to be a prominent source of cytokines upon contact with target cells (14). Such cytokine responses, together with cytolytic activity, may constitute an important component of early immune surveillance. While NK cell responses to soluble factors have been extensively studied (e.g., IFN- γ production by IL-12 and IL-18) (15), the molecular mechanisms that control cytokine and chemokine production during NK-target cell contact remain largely undefined.

Signaling by various surface receptors modulates the activity of diverse transcription factors, which in turn induce the reprogramming of gene transcription for cytokine and chemokine production. A key transcription factor for such

regulation is NF- κ B (16, 17). NK cells from patients deficient for NF- κ B components, such as NEMO and IKK β , demonstrate severe defects in IFN- γ production and cytotoxic function upon target cell recognition (18, 19), thus revealing the pivotal role of NF- κ B in NK cell effector functions via receptor stimulation. The signaling pathways leading to NF- κ B activation in NK cells have been characterized to some extent, but such studies are mostly confined to a few NK cell-activating receptors associated with immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptor molecules such as DAP12, FcR γ , and CD3 ζ (20, 21). These include NKp30 in humans and NK1.1, Ly49D, Ly49H, CD16, and NKG2D in mice. Unlike human NKG2D, murine NKG2D can associate with both DAP12 and DAP10 (22, 23). The signaling pathways downstream of ITAM-coupled receptors in NK cells are considered similar to those triggered by the antigen-specific receptors on B and T cells (9). In contrast, it remains unclear how the signaling cascades induced by non-ITAM-associated receptors (e.g., NKG2D, 2B4, DNAM-1) are coupled to NF- κ B activation. Furthermore, because of multiple receptor-ligand interactions that occur during NK-target cell contact, it is important to understand how signals from different NK cell receptors are coordinated to control NF- κ B activation.

Based on the requirement for cooperation among coactivation receptors to trigger effective cytokine production by resting NK cells, signaling by a single coactivation receptor may not suffice to activate NF- κ B. Instead, it may require integration of disparate signals from coactivation receptors for proper NF- κ B activation. In this study, we reveal that cooperative engagement of 2B4 with NKG2D

or DNAM-1 is required to achieve signaling competence for full NF- κ B activation. Furthermore, we uncover an unexpected checkpoint in NF- κ B activation by revealing a requirement for complementary signals to converge at the level of Vav1 and downstream NF- κ B p65 subunit. The pathophysiological relevance of this finding is supported by our identification of defective synergistic NF- κ B activation and NK cell responses centered on Vav1 in XLP1 NK cells, which lack functional SAP, a signaling molecule required for 2B4-mediated signaling.

III-3. Materials and Methods

III-3-1. Cells and reagents

Human blood samples from normal healthy donors and XLP1 donors were drawn for research purposes under a protocol approved by the institutional review board with informed consent. Patients with a confirmed SH2D1A gene mutation were included. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples by density gradient centrifugation (LSM lymphocyte separation medium; MP Biomedicals) and cryopreserved until processed. Human NK cells were purified from PBMCs by negative selection using the NK cell isolation kit (StemCell Technologies). These cells were 97–99% CD3-CD56+, as assessed by flow cytometry. The human NK cell line NKL (gift of M. Robertson) was cultured in RPMI1640 supplemented with 10% FBS, 1 mM sodium pyruvate, and 200 U ml⁻¹ recombinant IL-2 (rIL-2). NKL cells were rested in RPMI1640 supplemented with 5% FBS and 0.5 mM sodium pyruvate without rIL-2 for 24 h. P815 cells (American Type Culture Collection) were cultured in IMDM (Cellgro) supplemented with 10% FBS and 2mM L-glutamine. P815 cells are FcR+ and, upon incubation with Abs to NK cell activating receptors (e.g, NKG2D and 2B4), bind the Abs via the Fc-region. In doing so, they can activate NK cells through specific activating receptors in direct cell-cell contact. 721.221 cells (221; gift of J. Gumperz and P. Parham) and K562 cells (American Type Culture Collection) were maintained in IMDM (Cellgro) supplemented with 10% FBS. K562 cells are of the erythroleukemia type and known to express ligands for NKG2D, DNAM-1, and NKp30 receptors (44, 45). 221 cells

are an EBV-transformed B cell line, and their lysis by NK cells is associated with 2B4, NKp44, and NKp46 receptors (32, 46). K562 and 221 cells were used to validate the functional defects of XLP1 NK cells, given an impaired susceptibility of 221 cells to XLP1 NK cells likely due to the involvement of 2B4 in mediating lysis of the cells. K562-mb15-41BBL cell line (gift of D. Campana) for NK cell expansion were cultured in RPMI1640 supplemented with 10% FBS. Expression of human ligands for NK cell receptor in P815 cells has been described (47). 293T cells were cultured in DMEM (Cellgro) supplemented with 10% FBS and 2mM GlutaMAX (Gibco). The cells were free of mycoplasma contamination. All chemicals were from Calbiochem unless indicated otherwise.

III-3-2. Patient samples

Four patients with a confirmed mutation in the SH2D1A gene were included in this study. XLP1 patient samples (patients 1 and 2) were provided by Dr. C. Booth (48). Patient samples 3 and 4 were obtained from Dr. K. Nichols (49). Both patients 1 and 2 had macrodeletions in the SH2D1A gene. Patient 3 exhibited a T53I missense mutation in the SH2D1A protein and patient 4 had a germline deletion in the SH2D1A gene. The expression of SAP protein was absent in the NK cells obtained from 3 patients (patients 1, 2, and 4) and very low in patient 3's NK cells.

III-3-3. Abs

Abs for NK cell receptors and signaling molecules were obtained from the following sources: NKG2D (149810; R&D Systems); CD244/2B4 (C1.7; Beckman Coulter); isotype control mouse IgG1 (MOPC-21; Sigma); CD226/DNAM-1

(DX11), CD16 (3G8), CD94 (HP-3D9), pS32/36 I κ B α (39A1413), and actin (C4) (BD Biosciences); pY174 Vav1 (ab47282) and pS276 p65 (ab30623) (Abcam); Vav1 (H211), p65 (F-6), p50 (H-119), SAP (1D12), and I κ B α (C-21) (Santa Cruz); α -tubulin (GT114) (GeneTex); Vav1 (R775), pS536 p65 (93H1), pS176/180 IKK α / β (16A6), pS473 Akt (9271), pY202/204 Erk1/2 (9101), and TBP (44059) (Cell Signaling); c-Cbl (7G10) and T7 (AB3790) (Millipore). Goat F(ab')₂ anti-mouse IgG was obtained from Jackson ImmunoResearch. The fluorochrome-conjugated Abs were used in the flow cytometric analyses: α -pS536 p65-Alexa Fluor 488 (93H1) and isotype control rabbit IgG-Alexa Fluor 488 (DA1E) (Cell Signaling); α -CD3-PerCP (SK7), α -CD56-PE (NCAM16.2), α -CD107a-FITC (H4A3), α -IFN- γ -FITC (25723.11), α -CD16-PE (3G8), α -CD336/NKp44-PE (p44-8.1), α -NKp46-PE (9E2), α -CD226/DNAM-1-PE (DX11), and α -CD48-PE (TÜ145) (BD Bioscience); α -TNF- α -FITC (MAb11, eBioscience); α -CD244/2B4-PE (C1.7) and α -NKp30-PE (Z25) (Beckman Coulter); α -NKG2D-PE (149810), α -NKG2C-PE (134591), and α -ULBP1-PE (170818) (R&D Systems). CFSE, α -mouse IgG-Biotin, and Alexa Fluor 647-Streptavidin were obtained from Invitrogen for use in confocal microscopy. Mouse Fc block was done with anti-Fc γ RII/III (2.4G2) (BD Bioscience).

III-3-4. Cellular assays

IFN- γ (Pierce) and MIP-1 α (R&D Systems) production after stimulation of cells with beads coated with mAbs to NK receptors were determined by ELISA as described previously⁴. Briefly, beads for NK cell stimulation were prepared by incubating goat anti-mouse coated beads (4×10^7 ; Dynabeads M-450) with the indicated mAbs (3 μ g) in PBS containing 2% FBS for 1 h at 4°. After washing 3

times in PBS, 2% FBS, the beads (4×10^7) were incubated with rested NKL cells (4×10^7) in 500 μ L IMDM, 10% FBS for the indicated times at 37°. The cultures were rotated end-over-end during the stimulation, after which the supernatants were assayed.

Intracellular Ca^{2+} mobilization was measured by flow cytometry in cells labeled with Fluo-4 AM (Invitrogen) as described (34). Briefly, NK cells were labeled for 30 min at 30°C with dye-loading buffer [Hanks' balanced salt solution (HBSS) with 1% FBS, 2 μ M Fluo-4 AM, and 4 mM probenecid]. Cells were then washed twice, resuspended in HBSS, 1% FBS, and incubated with the indicated mAbs (10 μ g ml^{-1}) for 30 min on ice. Cells were washed twice and resuspended in HBSS containing 1% FBS, and transferred to flow cytometry analysis tubes. Cells were warmed for 5 min at 37° in a water bath, and placed on the flow cytometer. After 30 s of data acquisition, tubes were removed, and 4 μ g of cross-linking goat anti-mouse F(ab')₂ was added. Cells were mixed by vortexing, placed back on the flow cytometer (BD Bioscience), and events were acquired for a further 5 min. Data were analyzed with FlowJo software (Tree Star).

Degranulation of NK cells was assessed by CD107a expression on the cell surface and granzyme B release (BioLegend) as described (28). Briefly, primary rested NK cells were mixed with an equal number of K562 or 221 cells in the presence of anti-CD107a-FITC mAbs, spun down for 3 min at 30 \times g, incubated for 2 h at 37°C, and spun down again. The cell pellets were resuspended in FACS buffer (PBS with 2% FBS) and stained with anti-CD56-PE for 30 min in the dark at 4°C. Lymphocytes were gated on forward scatter/side scatter, and the CD107a expression on NK cells was analyzed by flow cytometry and FlowJo software. For granzyme B

release assay, rested NKL cells after transfection with control siRNA or p65-specific siRNA were stimulated with plate-immobilized mAbs to NKG2D and/or 2B4 for 2 h. Thereafter, granzyme B release into the supernatants were determined by ELISA.

Cytokine production by primary NK cells was determined by the intracellular expression of IFN- γ and TNF- α as described (14). PBMCs or primary rested NK cells were stimulated with an equal number of the indicated target cells for 1 h at 37°C. Thereafter, brefeldin A (GolgiPlug; BD Bioscience) was added, followed by an additional 5 h of incubation for a total of 6 h. The cells were then stained for surface markers with anti-CD56-PE and/or anti-CD3-PerCP mAbs for 30 min in the dark at 4°C. After washing the cells twice with FACS buffer, they were incubated in BD Cytofix/Cytoperm solution (BD Bioscience) for 20 min in the dark at 4°C. Before and after intracellular staining with anti-IFN- γ -FITC or anti-TNF- α -FITC mAb for 30 min in the dark at 4°C, the cells were washed twice with BD Perm/Wash buffer (BD Bioscience). The cells were then analyzed by flow cytometry gated on NK cells.

III-3-5. Receptor crosslinking and cell mixing experiments

For Ab-mediated crosslinking of NK receptors, NK cells were preincubated with isotype control mAb or mAbs specific for NK receptors (all at 10 $\mu\text{g ml}^{-1}$) for 30 min on ice. After washing unbound Abs with medium, NK cells were stimulated by crosslinking with 30 $\mu\text{g ml}^{-1}$ goat anti-mouse F(ab')₂ secondary Ab at 37°C for the indicated times. For stimulation of NK receptors mediated by plate-immobilized Abs, 96-well Costar EIA/RIA Stripwell plates were coated overnight at 4°C with isotype control mAb or mAbs specific for NK receptors (all at 10 μgml^{-1}). Then, NK

cells were added into NK receptor mAb-coated plates for the indicated times. For cell mixing experiments, NK and P815 target cells separately chilled on ice were mixed at an effector to target ratio of 1:1. Cells were incubated for 10 min on ice and then incubated at 37°C for the indicated times. Cells were moved to ice and then lysed for further analysis.

III-3-6. Assessment of NF- κ B activation by EMSA

Nuclear and cytoplasmic fractions were isolated from stimulated and unstimulated NKL cells using Nuclear Extract kit (Active Motif) according to the manufacturer's protocol. The amount of p65 in nuclear fraction and its DNA binding activity were measured by an immunoblotting and TransAM NF- κ B p65 ELISA kit (Active Motif) following the manufacturer's instruction, respectively.

III-3-7. NF- κ B reporter assay

NKL reporter cells (NKL- κ B-GFP) that express GFP under the control of NF- κ B transcription response elements (TRE) were generated by transducing NKL cells with lentiviral κ B-GFP construct (System Biosciences). Lentiviral particles were produced by simultaneous transfection of 293TN packaging cell line (System Biosciences) with pGreenFire-NF- κ B-EF1-Puro vector and pPACKH1 packaging plasmid mix (System Biosciences). NKL cells were transduced with lentivirus supernatant that encodes κ B-GFP in the presence of 10 μ g ml⁻¹ polybrene and 200 U ml⁻¹ rIL-2 and then selected with 1 μ g ml⁻¹ puromycin (2 days after transduction). Thereafter, NKL transductants showing GFP upregulation upon TNF- α treatment

were further selected by FACS-sorting and grown as pure cultures. To assess NF- κ B activation, NKL- κ B-GFP cells were stimulated with TNF- α or plate-immobilized mAbs specific for NK receptors, and GFP expression in the reporter NKL cells was analyzed by flow cytometry.

III-3-8. RNA interference

NKL cells were transfected with 300 pmoles of siRNA with the Amaxa Nucleofector II system. A total of 1.2×10^6 cells were resuspended in 100 μ l of Amaxa kit solution V (Lonza), mixed with siRNA, and immediately transfected with program O-017. For a total of 48 h incubation at 37°C, cells were rested for the last 24 h and then assayed as indicated. For knockdown in primary NK cells, 1.5×10^6 expanded cells were resuspended in 100 μ l of Amaxa kit solution for human macrophage, mixed with 300 pmol of siRNA, and transfected with program X-001. Thereafter, cells were incubated for 36 h in the presence of IL-2 (200 U ml⁻¹), rested for 12 h, and then assayed as indicated. The siRNAs specific for NF- κ B p65 were part of TriFECTa Dicer-substrate kit obtained from Integrated DNA Technologies (IDT) and the following siRNA sequences were used: p65, 5'-GGA GUA CCC UGA GGC UAU AAC UCG C-3' (sense) and 5'-GCG AGU UAU AGC CUC AGG GUA CUC CAU-3' (antisense) or 5'-GGA CAU AUG AGA CCU UCA AGA GCA T-3' (sense) and 5'-AUG CUC UUG AAG GUC UCA UAU GUC CUU-3' (antisense). The siRNAs used to knockdown of Vav1 and c-Cbl were described previously⁴ and the sequences are as follows: Vav1, 5'-CGU CGA GGU CAA GCA CAU UdTdT-3' (sense) and 5'-AAU GUG CUU GAC CUC GAC GdTdT-3' (antisense); c-Cbl: 5'-CCU CUC UUC CAA GCA CUG AdTdT-3' (sense) and 5'-UCA GUG CUU GGA

AGA GAG GdTdT-3' (antisense). ON-TARGETplus SMARTpool siRNAs specific for Vav1 (L-003935), c-Cbl (L-003003) were also obtained from Dharmacon. The siRNA specific for SAP were obtained from IDT and the sequences are as follows: SAP, 5'-GCU GUA UCA CGG UUA CAU UUA UAC A-3' (sense) and 5'-UGU AUA AAU GUA ACC GUG AUA CAG CAC-3' (antisense). siRNA specific for SAP (SI00036561) were also obtained from Qiagen. ON-TARGETplus SMARTpool siRNAs specific for Akt1 (L-003000) or Erk2 (L-003555) were obtained from Dharmacon. A second set of siRNAs for Akt1 and Erk2 was used, with the following sequences: Akt1, 5' - GGA CAG AGG AGC AAG GUU UdTdT-3' (sense) and 5'-AAA CCU UGC UCC UCU GUC CdTdT-3' (antisense); Erk2: 5'-GGG UUC CUG ACA GAA UAU GdTdT-3' (sense) and 5'-CAU AUU CUG UCA GGA ACC CdTdT-3' (antisense). Similar results were obtained with either one of the siRNAs used. The results shown in this paper are those obtained with the former set of siRNA oligonucleotides. The negative siRNA controls were obtained from IDT and Dharmacon.

III-3-9. *Ex vivo* expansion of NK cells

Due to the limited supply of patient NK cells, NK cells from XLP1 patients were expanded to study molecular signals for NF- κ B activation and effector functions. NK cells from normal donors were also expanded and, after a period of rest, reproduced the synergistic increase in effector functions and signaling following stimulation with NKG2D and 2B4. Primary NK cells obtained from normal or XLP1 donors were expanded by stimulation with the K562-mb15-41BBL cell line, as previously described (50, 51) with slight modifications. 1.5×10^6 PBMCs were

cultured with 1×10^6 100 Gy gamma ray-irradiated K562-mb15-41BBL feeder cells in stem cell growth medium (SCGM) (CellGenix) supplemented with 10% FBS and 10 U ml^{-1} rIL-2. The medium was changed every 2 days and replaced with fresh medium containing 10 U ml^{-1} rIL-2. After 1 week of coculture, residual T cells were depleted using the CD3⁺ selection kit (StemCell Technologies). The remaining cells were further expanded for an additional 2 weeks in SCGM supplemented with 10% FBS, 100 U ml^{-1} rIL-2, and 5 ng mL^{-1} rIL-15. The resulting cell population was 97 to 99% CD3-CD56⁺, as assessed using flow cytometry.

III-3-10. DNA constructs and transfections

Given a difficulty in detecting p65 phosphorylation at serine 276 (52), the specificity of the antibody against pS276 p65 (ab30623) was confirmed (Fig. 81). The plasmids encoding T7-RelA (#21984) or T7-RelA S276A (#24153) were obtained from Addgene and verified by sequencing. 0.5×10^6 293T cells were cultured for 18 h and then transfected with 2 μg of plasmid DNA using XtremeGENE 9 (Roche). Medium was changed after 24 h posttransfection and transfected cells were assayed after another 24 h.

III-3-11. Immunoblotting

The stimulated NK cells were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM NaVO₃, 50 mM NaF, 1mM PMSF, and protease inhibitor cocktail [Thermo]) for 30 min on ice. Cell debris, including the nuclei, was removed by centrifugation,

and the supernatants were recovered. The protein concentration of the cell lysates was determined using the BCA protein assay kit (Pierce). Lysates were resuspended in 1× NuPAGE LDS sample buffer (Invitrogen) containing 50mM dithiothreitol (DTT), and further incubated for 10 min at 70°C. Equal amounts of protein for each sample were resolved on 8% Tris-HCl gel or 4-20% Tris-HEPES gel (Thermo) and subsequently transferred onto PVDF membranes (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 20% [v v⁻¹] methanol). The membranes were blocked with 5% BSA or skim milk in TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 h and subsequently incubated with primary Abs and then with the HRP-conjugated secondary Abs. Blots were developed using SuperSignal West Pico and detected using LAS-4000 (Fujifilm). Images have been cropped for presentation.

III-3-12. Cell conjugation and immunofluorescence

To examine NF-κB p65 translocation into the nucleus in the individual NKL cells that were conjugated with target cells, NKL cells were first stained with 10 μM CFSE (Invitrogen) for 15 min at 37°C, washed, and resuspended in IMDM supplemented with 5% FBS. P815 cells were incubated with 10 μg ml⁻¹ isotype control mAb or mAbs specific to NK receptors for 20 min at room temperature (RT), spun down, and resuspended in IMDM containing 5% FBS. Thereafter, CFSE-loaded NKL cells were conjugated to the P815 target cells at a 2:1 E:T ratio for 30 min at 37°C and then allowed to adhere to poly-L-lysine-coated slide glass (Sigma) for 15 min at 37°C. Slides were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min at RT and in cold methanol for 10 min at -20°C. The cells were then permeabilized with 0.1% Triton X-100 (Sigma) and 0.1% sodium citrate (Sigma)

in PBS for 3 min at 4°C and blocked with PBS containing 1% BSA and 1% goat serum for 30 min. Slides were incubated with primary Ab to p65 (1:100, F-6; Santa Cruz) for 90 min, α -mouse IgG-Biotin (1:100; Invitrogen) for 40 min, Alexa Fluor 647-Streptavidin (1:50; Invitrogen) for 30 min in PBS containing 1% BSA and 0.5% Triton X-100, and then DAPI (300 nM; Molecular Probes) for 2 min. All slides were incubated at RT and washed 3 times with PBS. The slides were mounted with ProLong Gold antifade reagent (Molecular Probes) and 0.13–0.16 mm coverslips (Marienfeld). The cells were imaged using the Zeiss LSM710 laser-scanning Confocal Microscope (Carl Zeiss) and analyzed using the MetaMorph software (Molecular Devices). NKL-target cell conjugates were verified by the presence of a CFSE-stained cell that had conjugated to an unstained cell.

III-3-13. Real-time PCR

To assess the expression of genes related to NK cell effector functions, total RNA was isolated using the RNeasy kit (QIAGEN). cDNA was synthesized from 1 μ g RNA using the ReverTra Ace qPCR RT kit (Toyobo) according to the manufacturer's instructions. Real-time PCR amplification and analysis were conducted using the SYBR Green Realtime PCR Master Mix (Toyobo) and LightCycler 480 Real-Time PCR System (Roche Diagnostics). The PCR conditions were as follows: preheating for 10 min at 95°C, 40 cycles of 95°C (30 s), 60°C (30 s) and 72°C (30 s). Melting curve analysis was done using the default settings of the device. RNA levels were normalized to β -actin expression using the $\Delta\Delta C_t$ method. Primer sequences for real-time PCR are described in the Table 1.

III-3-14. Flow cytometric analysis of p65 phosphorylation

Purified human primary NK or NKL cells were incubated with isotype control mAb or mAbs specific to NK receptors on ice for 30 min. After being washed with medium, NK cells were stimulated by receptor cross-linking with goat anti-mouse F(ab')₂ secondary Ab at 37°C for 5 min. The cells were washed with DPBS and fixed with 4% paraformaldehyde at 37°C for 10 min. The fixed cells were then permeabilized with 90% methanol on ice for 30 min and blocked with 0.5% BSA at room temperature (RT) for 10 min. Cells were analyzed for p65 phosphorylation using flow cytometry after staining with Alexa Fluor 488-conjugated anti-pS536 p65 Ab (93H1) or isotype control rabbit IgG (DA1E) (Cell Signaling).

III-3-15. Statistical analyses

Each graph was generated from at least three independent experiments. Results are presented as the mean \pm s.d. or s.e.m. Statistical analyses were conducted by two-tailed Student's *t*-test using the GraphPad Prism software.

III-3-16. Data availability

The data that support the findings of this study using patient samples are available on request from the corresponding author [H.S.K.]. The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

III-4. Results

III-4-1. Synergistic activation of NF- κ B by NKG2D and 2B4

Various stimulatory receptors trigger distinct signaling pathways that lead to NF- κ B activation (24). Such diverse NF- κ B-activating stimuli, however, frequently converge on the I κ B kinase (IKK) complex, and signaling cascade downstream of IKK appears to be well-conserved among most NF- κ B activation pathways. This includes the phosphorylation and degradation of the inhibitor of κ B (I κ B) family, which releases bound NF- κ B dimers for nuclear translocation and target gene transcription. So far, little is known about the mechanisms that couple non-ITAM-associated receptors to NF- κ B activation during the triggering of natural cytotoxicity by NK cells. Thus, we first assessed NF- κ B activation after stimulating NK cells via NKG2D, 2B4, or both. Stimulation of human NK cell line NKL with NKG2D or 2B4 alone resulted in detectable but weak phosphorylation of IKK and Erk, whereas coengagement led to their synergistic phosphorylation (Fig. 29). Such an increase was distinct from Akt phosphorylation, which is downstream of PI3K activation via NKG2D but not 2B4 (Fig. 29). Likewise, phosphorylation of the downstream NF- κ B p65 subunit (pS536-p65) resembled that of IKK. Such synergistic phosphorylation was evident at 5 min and decreased thereafter, which coincided with the phosphorylation and degradation of I κ B α that links IKK activation and p65 nuclear translocation (Fig. 30). We next performed microscopy experiments to assess nuclear translocation of p65 in individual NKL-target cell conjugates. To facilitate NKL cell identification, they were labeled with CFSE and

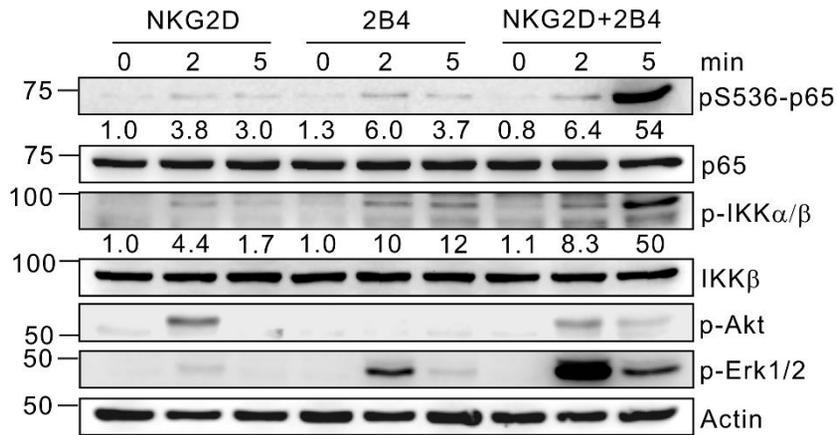


Fig. 29. Synergistic phosphorylation of NF- κ B by NKG2D and 2B4 co-engagement.

NKL cells rested in the absence of IL-2 for 24 h were stimulated with NKG2D and/or 2B4 by receptor crosslinking for the indicated time. Cell lysates were immunoblotted with Abs to phospho-p65 at serine 536 (pS536), p65, phospho-IKK α/β at serine 176/180 (pS176/180), IKK β , phospho-Akt at serine 473 (pS473), phospho-Erk1 and 2, or actin. The normalized intensities of the phosphorylated p65 and IKK α/β relative to their total forms are presented. Data are representative of at least three independent experiments.

conjugated with unlabeled

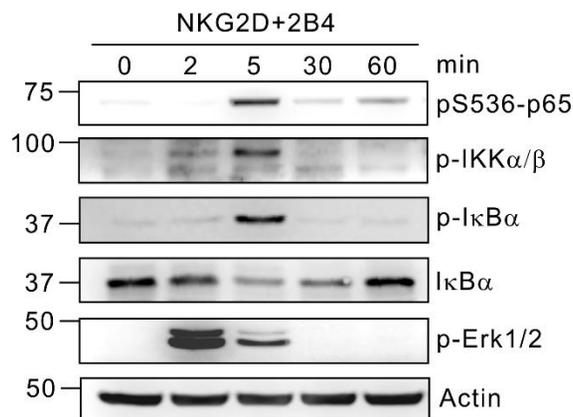


Fig. 30. Kinetics of phosphorylation of NF- κ B signaling pathway by NKG2D and 2B4 co-engagement.

Rested NKL cells were treated as in (a) to stimulate NKG2D and 2B4 for the indicated time. Lysates were immunoblotted for phospho-p65, phospho-IKK α/β , phospho-I κ B α at serine 32/36 (pS32/36), I κ B α , phospho-Erk1/2, or actin. Data are representative of at least three independent experiments.

P815 target cells. In conjugated NKL cells, little or weak nuclear translocation of p65 was observed after stimulation with NKG2D or 2B4 alone (Fig. 31). In contrast, significant nuclear translocation of p65 was detected in conjugated NKL cells following NKG2D and 2B4 coengagement (Fig. 31). Supporting this, analysis of subcellular fractions revealed a substantial increase in nuclear p65 and concomitant decrease in cytoplasmic p65 in NKL cells stimulated with NKG2D and 2B4 but neither receptor alone (Fig. 32). Further, the amount of p65 bound to oligonucleotide containing consensus NF- κ B-binding site was significantly increased in the nuclear extracts of NKL cells following NKG2D and 2B4 coengagement (Fig. 33). Finally, to evaluate the transcriptional activity of NF- κ B, we generated NKL reporter (NKL- κ B-GFP) cells that express GFP under the control of NF- κ B transcription response elements (TRE). Proper functioning of the reporter cells was demonstrated by increased GFP expression following TNF- α treatment and its abrogation by prior treatment with BAY11-7082 that inhibits NF- κ B activation (Fig. 34). Compared with either receptor alone, NKG2D and 2B4 coengagement induced an apparent and synergistic increase in the proportion of cells expressing GFP (Fig. 35). Thus, via comprehensive analyses of conserved steps in the NF- κ B activation pathway, we conclude that robust NF- κ B activation is not achieved by stimulation with NKG2D or 2B4 alone, but instead relies on the integration of distinct signals from NKG2D and 2B4.

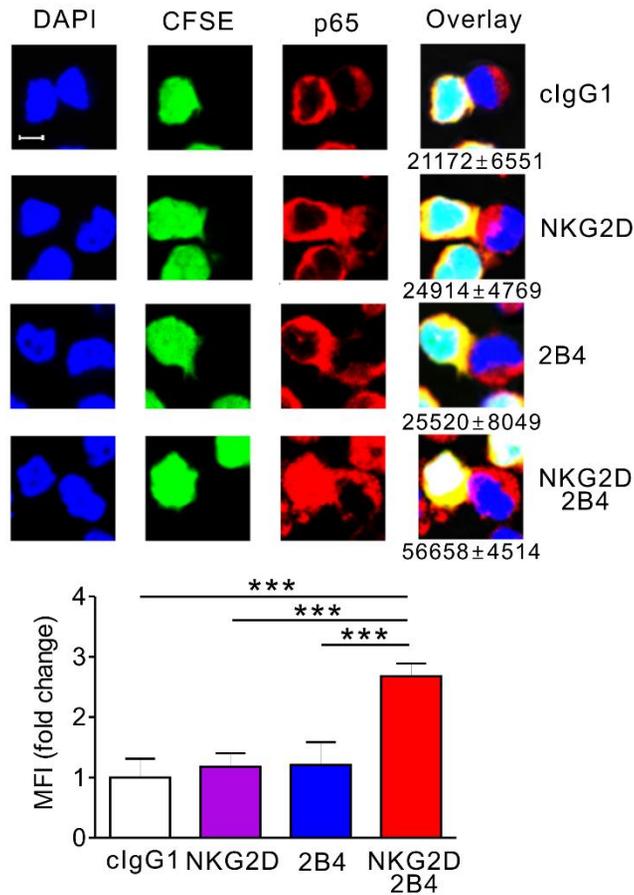


Fig. 31. Immunofluorescence for nuclear translocation of p65 by NKG2D and 2B4 co-engagement.

Representative confocal images (top) of conjugates between rested NKL cells loaded with CFSE (green) and P815 target cells as indicated. Conjugates were fixed, permeabilized, and stained with DAPI (blue) and mAb to p65, anti-mouse IgG-Biotin followed with Alexa Fluor 647 (red)-Streptavidin. The number beneath the overlay image is the mean nuclear fluorescence intensity (MFI) ± s.d. of p65 from ≥ 50 NKL-target cell conjugates. Statistical bar charts (bottom) for MFI of p65 in the nucleus are represented as fold change. Values represent mean ± s.d. Scale bar, 5 μm. ***P < 0.001 (two-sided Student's t-test). Data are representative of at least three independent experiments.

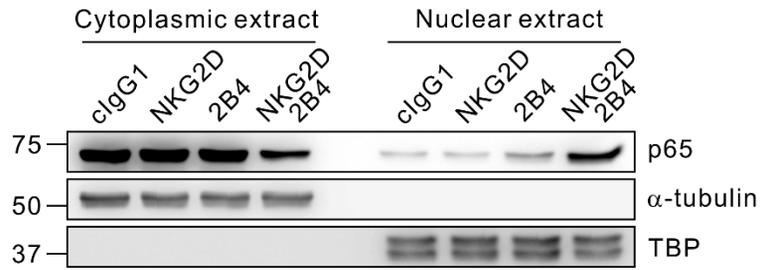


Fig. 32. Subcellular fractionation for nuclear translocation of p65 by NKG2D and 2B4 co-engagement.

Rested NKL cells were stimulated with plate-immobilized mAbs to NKG2D and/or 2B4 for 1 h. Equal amounts of protein from cytoplasmic and nuclear extracts were immunoblotted with mAb to p65. Data are representative of at least three independent experiments.

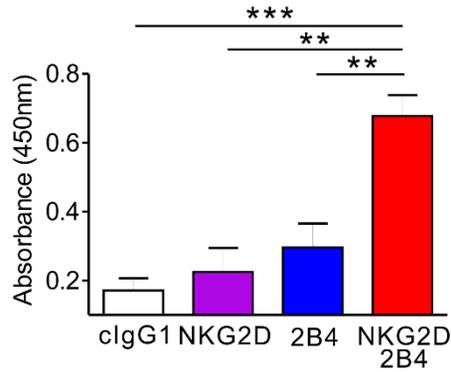


Fig. 33. DNA binding nuclear p65 after NKG2D and 2B4 co-engagement.

Nuclear extracts collected as in Fig. 32. were added into a 96-well plate immobilized with double-stranded oligonucleotide containing the consensus NF- κ B binding sequence. The amount of p65 bound to the oligonucleotide was measured by colorimetric assay. Values represent mean \pm s.d. **P < 0.01; ***P < 0.001 (two-sided Student's t-test). Data are representative of at least three independent experiments.

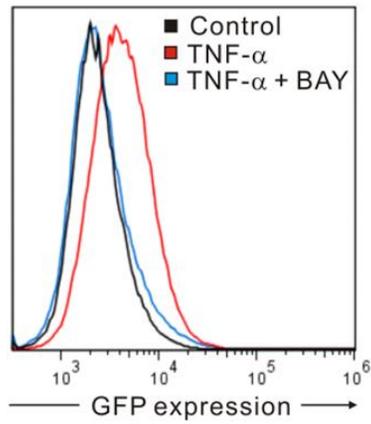


Fig. 34. GFP expression in NKL- κ B-GFP cells is NF- κ B-dependent.

Rested NKL cells transduced with the κ B-GFP reporter construct were treated with 10 ng/mL TNF- α for 6 h after pretreatment with the vehicle or NF- κ B inhibitor BAY11-7082 (1 μ M) for 1 h. GFP expression in NKL- κ B-GFP cells was analyzed using flow cytometry.

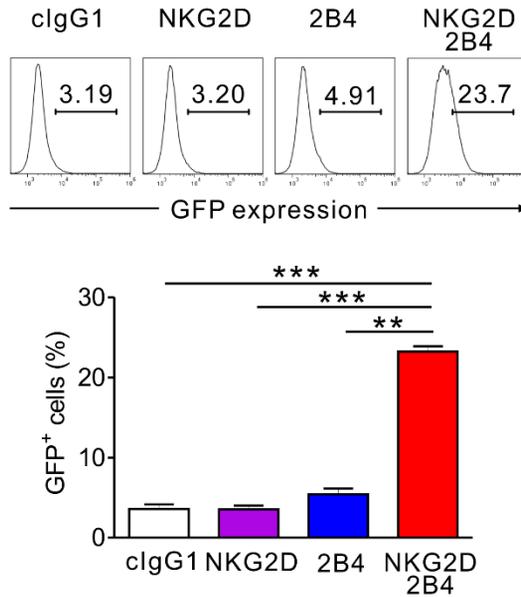


Fig. 35. Transcriptional activity of NF- κ B after NKG2D and 2B4 co-engagement.

Rested NKL cells transduced with a κ B-GFP reporter construct were stimulated with plate-immobilized mAbs to NKG2D and/or 2B4 for 6 h. GFP expression in NKL- κ B-GFP cells was analyzed by flow cytometry, and representative result (top) and statistical bar charts (bottom) are shown. Values represent mean \pm s.d. **P < 0.01; ***P < 0.001 (two-sided Student's t-test). Data are representative of at least three independent experiments.

III-4-2. NF- κ B is required for NK cell functions by NKG2D and 2B4

Given NF- κ B as a transcription factor important for gene regulation, we assessed gene expression after stimulating NK cells with NKG2D, 2B4, or both. Examination of a profile of 20 genes encompassing cytokines, chemokines, cytolytic pathway, death receptor pathway, NF- κ B pathway, apoptosis, IL-2 receptor and cytotoxic granule exocytosis revealed a synergistic induction of diverse genes related to NK cell effector functions (Fig. 36), correlating with the increase in NF- κ B activation. Chemokine expression appears to be preferentially triggered by engagement of a single receptor (especially NKG2D), corroborating a recent study of the requirement for less stimulation to induce chemokine production compared to cytokine production or degranulation (14). Nonetheless, NKG2D and 2B4 coengagement still induced higher levels of chemokine and cytokine gene expression.

Among the major genes induced by NF- κ B are those encoding cytokines and chemokines. The synergy-dependent activation of NF- κ B (Fig. 29-35) and mRNA expression of IFN- γ and MIP-1 α (4) (Fig. 36) prompted us to test whether NF- κ B is required for NK cells to produce cytokines and chemokines following NKG2D and 2B4 engagement. The synergistic production of IFN- γ and MIP-1 α by NKL cells was diminished in a dose-dependent manner following BAY11-7082 treatment (Fig. 37). To directly probe the role of NF- κ B, we silenced the expression of NF- κ B p65 subunit, using small interfering RNA (siRNA) (Fig. 38). p65 knockdown caused marked reductions in mRNA expression of IFN- γ , TNF- α , MIP-1 α/β , granzyme B, and I κ B α induced by NKG2D and 2B4 coengagement (Fig. 39), confirming the

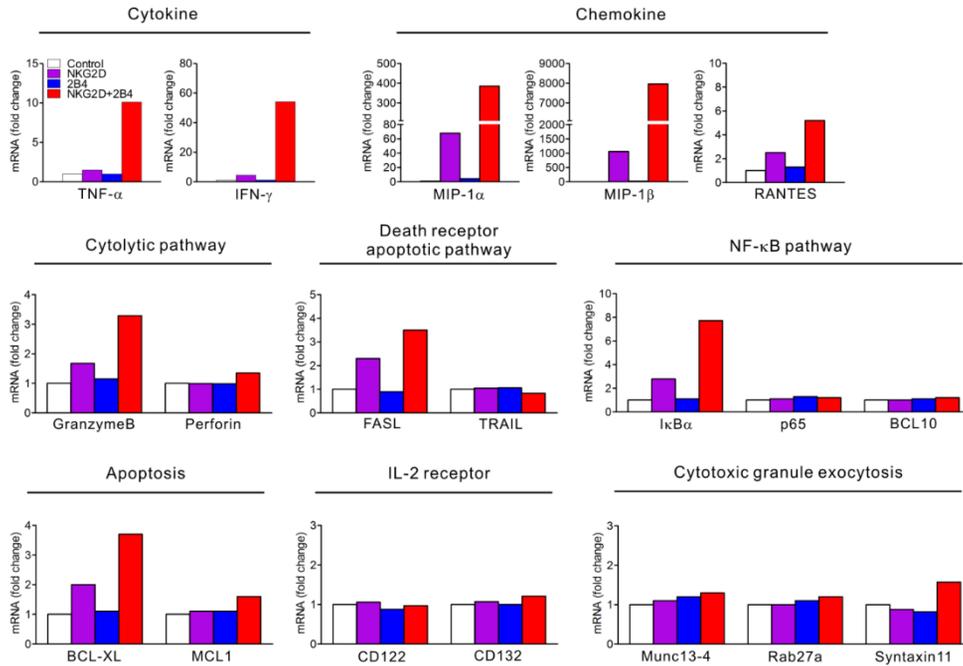


Fig. 36. Profile of gene expression related to NK cell function during coactivation.

Rested NKL cells were stimulated with NKG2D and/or 2B4 for 3 h. Thereafter, total RNA was prepared from cells, reverse transcribed, and analyzed by real-time PCR with primers specific for TNF- α , IFN- γ , MIP-1 α , MIP-1 β , RANTES, Granzyme B, Perforin, FASL, TRAIL, I κ B α , p65, BCL10, BCL-XL, MCL1, CD122, CD132, Munc13-4, Rab27a, or Syntaxin11. The relative mRNA levels were determined by real-time PCR and normalized to β -actin mRNA. Data are representative of three independent experiments.

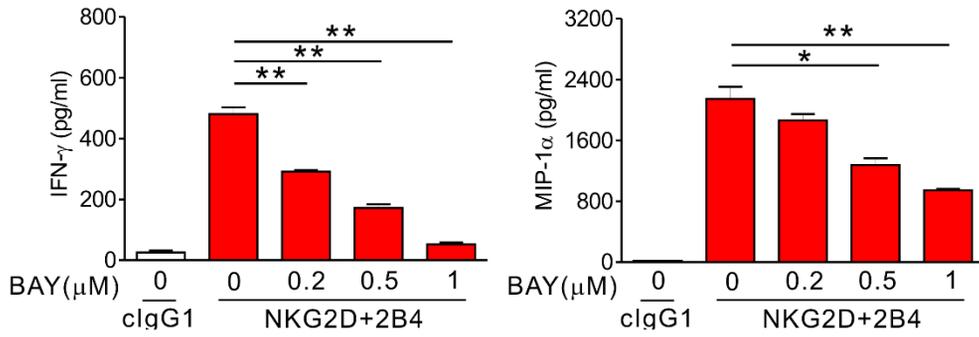


Fig. 37. BAY11-7082 inhibits cytokine and chemokine secretion of NKL cells by NKG2D and 2B4 coactivation.

Rested NKL cells were pretreated with a NF-κB inhibitor BAY11-7082 at the indicated dose for 1 h and then stimulated with both NKG2D and 2B4 for 8 h. Thereafter, IFN-γ and MIP-1α in the supernatants were measured by ELISA. Values represent mean ± s.d. *P < 0.05; **P < 0.01 (two-sided Student's t-test). Data are representative of at least three independent experiments.

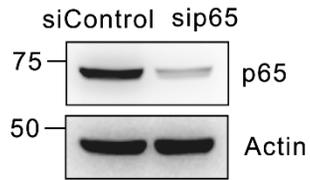


Fig. 38. Knockdown of p65 in NKL cells with p65-specific siRNA.

NKL cells were transfected with 300 pmoles of control siRNA or siRNA specific for p65. After 24 h, the cells were rested for another 24 h, and lysates were immunoblotted for p65 and actin. Data are representative of at least three independent experiments.

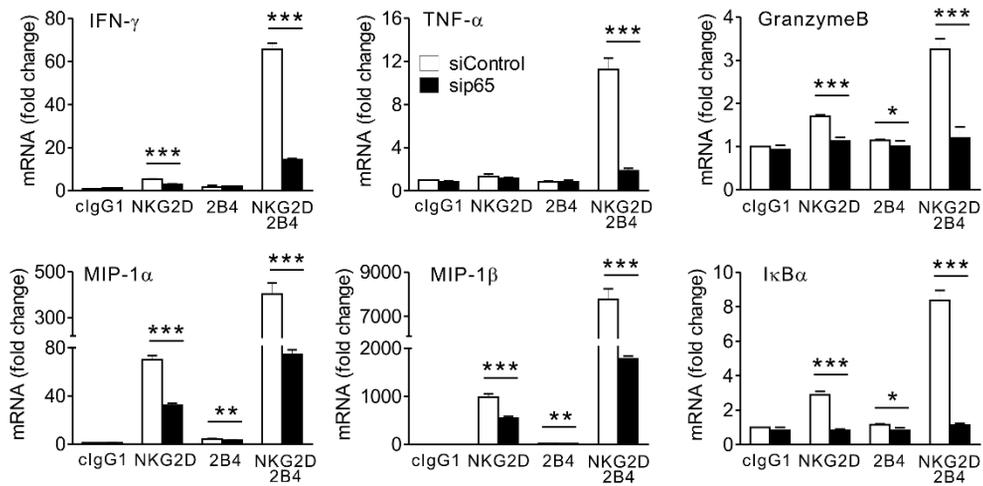


Fig. 39. p65-specific siRNA inhibits cytokine, chemokine, granzyme B, and I κ B α mRNA expression of NKL cells by NKG2D and 2B4 coactivation.

Rested NKL cells transfected with control siRNA or p65-specific siRNA were stimulated with NKG2D and/or 2B4 for 3 h. Thereafter, total RNA was prepared from cells, reverse transcribed, and the relative mRNA levels of IFN- γ , TNF- α , granzyme B, MIP-1 α , MIP-1 β , and I κ B α were determined by real-time PCR and normalized to β -actin mRNA. Values represent mean \pm s.d. * P < 0.05; ** P < 0.01; *** P < 0.001 (two-sided Student's t -test). Data are representative of at least three independent experiments.

dependence of their transcription on NF- κ B. Similarly, the synergistic production of IFN- γ and MIP-1 α was significantly reduced by p65 knockdown (Fig. 40). To confirm this finding in the context of physiological receptor-ligand interactions, we stimulated NKL cells with P815 cells expressing ULBP1 (a ligand for NKG2D) and/or CD48 (a ligand for 2B4). Consistently, synergistic production of IFN- γ and MIP-1 α following physiological stimulation was significantly diminished by p65 knockdown (Fig. 41).

Given the reports showing defective cytotoxicity of NK cells from patients with NF- κ B deficiency (18, 19), we next assessed whether NF- κ B is also required for cytotoxic degranulation of NK cells. The synergistic increase in degranulation, as assessed by granzyme B release, was significantly decreased by p65 knockdown (Fig. 42). Collectively, NF- κ B could play an indispensable role in the production of cytokines and chemokines, as well as the release of granzyme B by NK cells via non-ITAM-associated receptors NKG2D and 2B4.

We next determined whether the findings obtained from NKL cells were applicable to primary NK cells. NF- κ B activation was assessed using purified NK cells on a per-cell basis by flow cytometry-based analysis of p65 phosphorylation. Similar to experiments using NKL cells (Fig. 43), the proportion of responding cells synergistically increased following combined stimulation of primary NK cells with NKG2D and 2B4 (Fig. 44). NKG2D and 2B4 coengagement also led to synergistic increase in the proportion of NK cells expressing IFN- γ or TNF- α (Fig. 45), which was diminished after BAY11-7082 treatment (Fig. 46). BAY11-7082 did not significantly affect the viability of NKL cells and primary NK cells, as assessed by

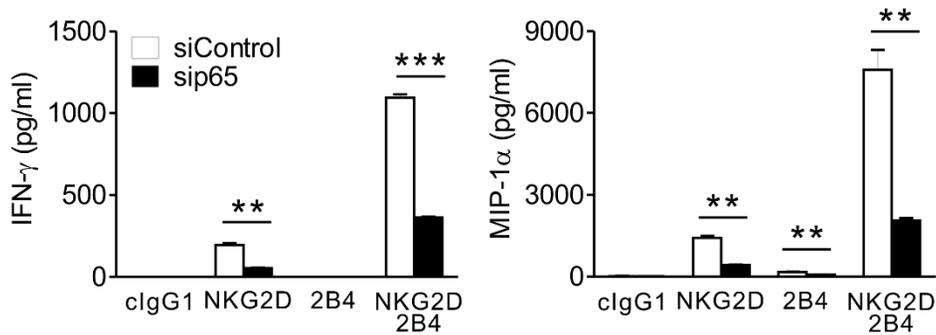


Fig. 40. p65-specific siRNA inhibits cytokine and chemokine secretion of NKL cells by NKG2D and 2B4 coactivation.

Rested NKL cells transfected with control siRNA or p65-specific siRNA were stimulated as in Fig. 39 for 8 h. IFN- γ and MIP-1 α in the supernatants were measured by ELISA. Values represent mean \pm s.d. ** $P < 0.01$; *** $P < 0.001$ (two-sided Student's t -test). Data are representative of at least three independent experiments.

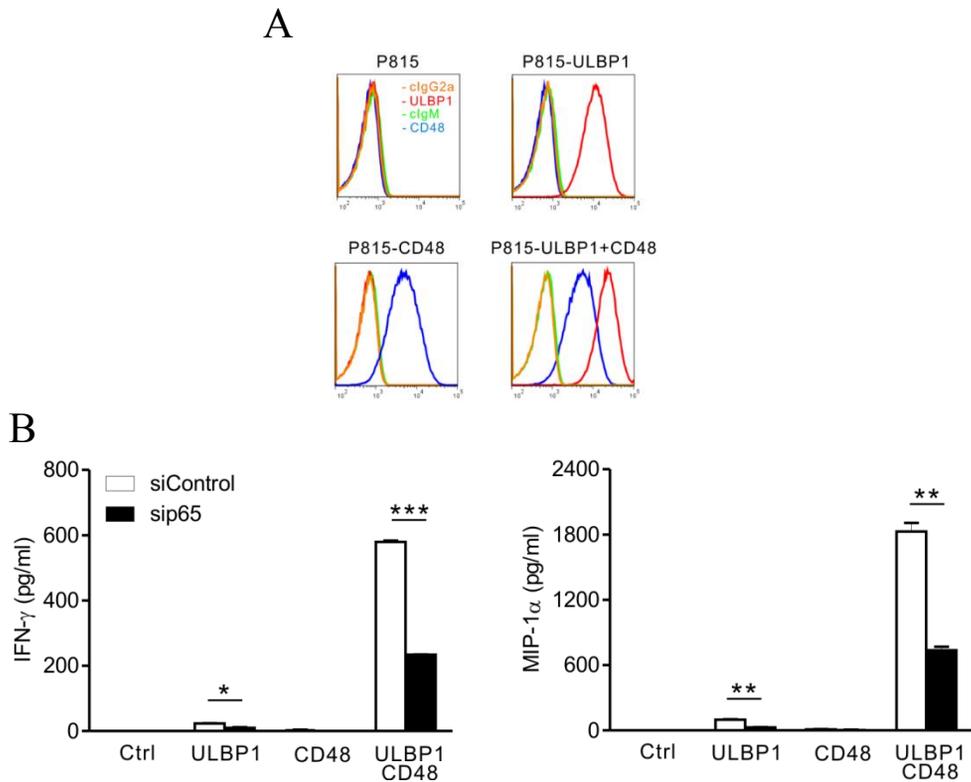


Fig. 41. IFN- γ and MIP-1 α production by NKG2D and 2B4 ligands is NF- κ B-dependent.

(A) Expression of NKG2D and 2B4 ligands on transfected P815 cells. P815, P815-ULBP1, P815-CD48, and P815-ULBP1+CD48 cells were stained with isotype control mAbs, or mAbs to ULBP1 and CD48.

(B) Cytokine release assays with rested NKL cells transfected with control siRNA or p65-specific siRNA and stimulated with P815 cells expressing ULBP1 and/or CD48. After incubation for 6 h, IFN- γ (left) and MIP-1 α (right) in the supernatants were measured by ELISA. Values represent mean \pm s.d. * P <0.05; ** P <0.01; *** P <0.001 (two-sided Student's t -test). Data are representative of three independent experiments.

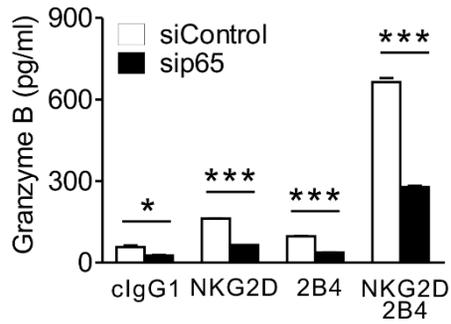


Fig. 42. p65-specific siRNA inhibits Granzyme B secretion of NKL cells by NKG2D and 2B4 coactivation.

Rested NKL cells that were transfected with control siRNA or p65-specific siRNA and stimulated with NKG2D and/or 2B4 for 2 h were used in a granzyme B release assay. Granzyme B in the supernatants was measured by ELISA. Error bars represent the s.d. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-sided Student's *t*-test). Data are representative of at least three independent experiments.

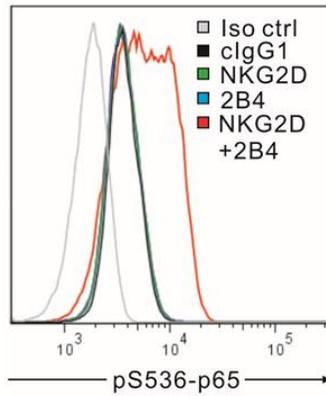


Fig. 43. The proportion of cells showing p65 phosphorylation is synergistically increased by NKG2D and 2B4 coactivation.

Phosphorylation of the NF- κ B p65 subunit in rested NKL cells that were stimulated with NKG2D and/or 2B4 by receptor crosslinking for 5 min. The cells were fixed, permeabilized, stained with Alexa Fluor 488-conjugated isotype control mAb or mAb specific to phospho-p65 at serine 536, and analyzed using flow cytometry.

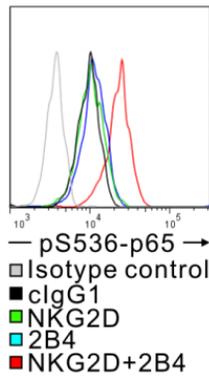


Fig. 44. Phosphorylation of p65 in primary NK cells by NKG2D and 2B4 coactivation.

Phosphorylation of NF- κ B p65 subunit in primary rested NK cells that were stimulated with NKG2D and/or 2B4 as in Fig. 29 for 5 min. Cells were fixed, permeabilized, stained with Alexa Fluor 488-conjugated isotype control mAb or mAb to phospho-p65 at serine 536, and analyzed by flow cytometry. Data are representative of at least three independent experiments.

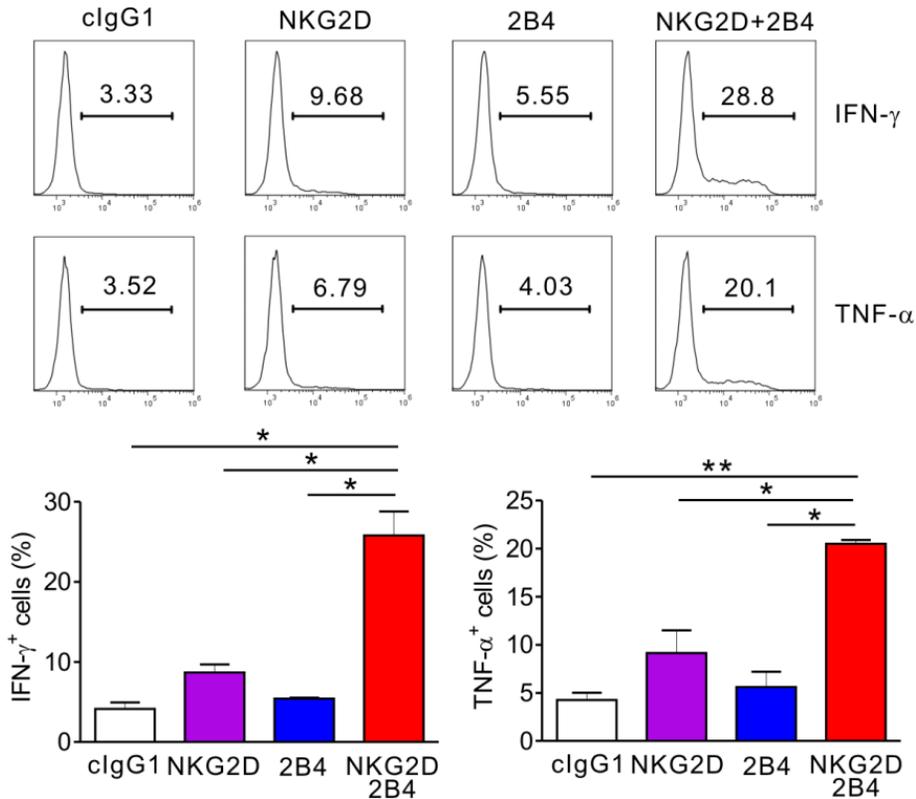


Fig. 45. The proportion of cells showing cytokine production by NKG2D and 2B4 coactivation is synergistically increased.

Frequency of NK cells that displayed IFN- γ or TNF- α expression after stimulation of PBMCs with P815 target cells as indicated. After incubation for 6 h, cells were stained for surface markers with mAbs to CD3 and CD56. Cytokine production by NK cells was measured by intracellular expression of IFN- γ or TNF- α in CD3-CD56⁺ NK cells. Representative result (top) and statistical bar charts (bottom) are shown. Values represent mean \pm s.e.m. * P < 0.05; ** P < 0.01 (two-sided Student's t -test). Data are representative of at least three independent experiments. Statistical bar charts show pooled data from three experiments.

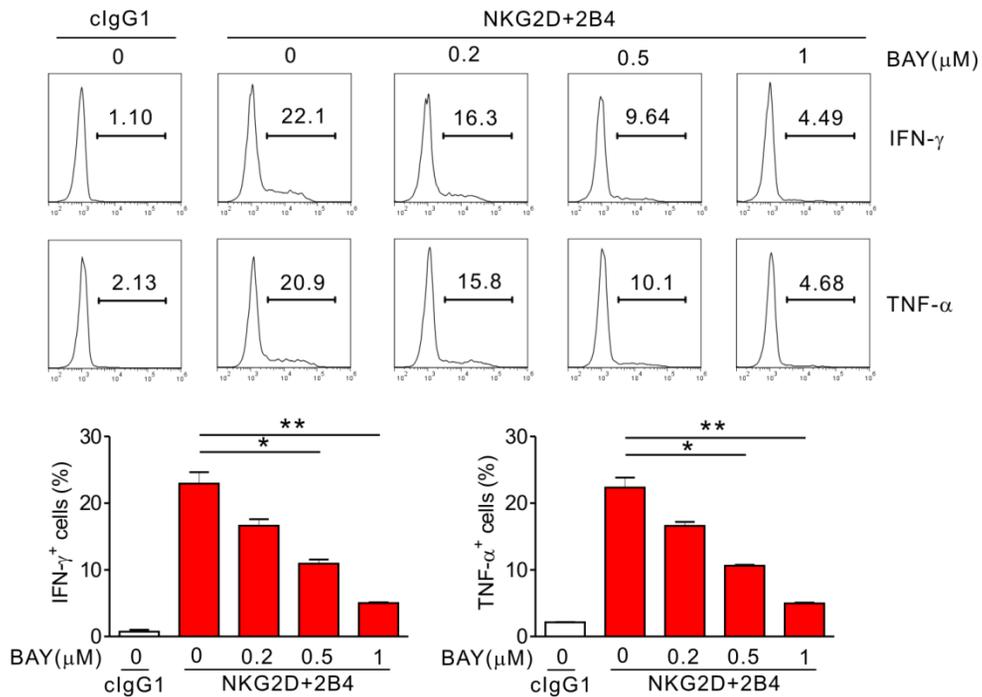


Fig. 46. The proportion of cells showing cytokine production by NKG2D and 2B4 coactivation is inhibited by BAY11-7082.

Frequency of NK cells that displayed IFN- γ or TNF- α expression after pretreatment of PBMCs with BAY11-7082 at the indicated dose for 1 h and then stimulation with P815 target cells as indicated in the presence of the inhibitor. After incubation for 6 h, cells were stained and analyzed by flow cytometry as in Fig 45. Representative result (top) and statistical bar charts (bottom) are shown. Values represent mean \pm s.e.m. * $P < 0.05$; ** $P < 0.01$ (two-sided Student's t -test). Data are representative of at least three independent experiments. Statistical bar charts show pooled data from three experiments.

annexin-V/PI staining (Fig. 47). Collectively, these results suggest that NKG2D and 2B4 coactivation is required to overcome a threshold for NF- κ B activation, leading to synergistic cytokine production by NK cells.

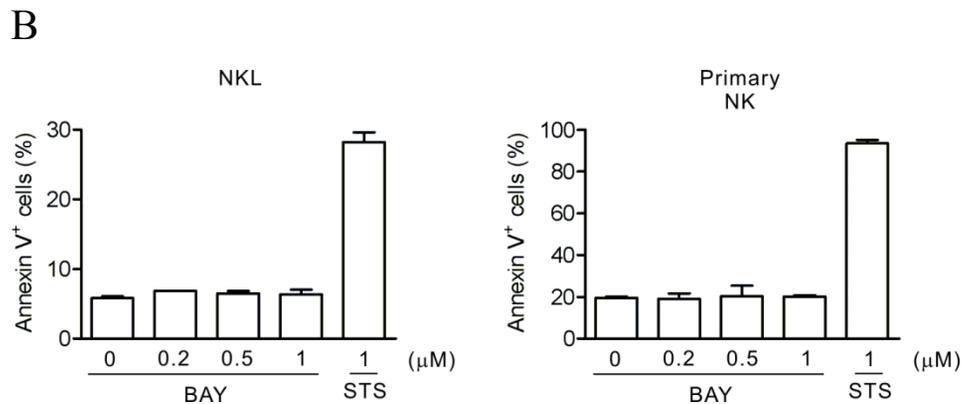
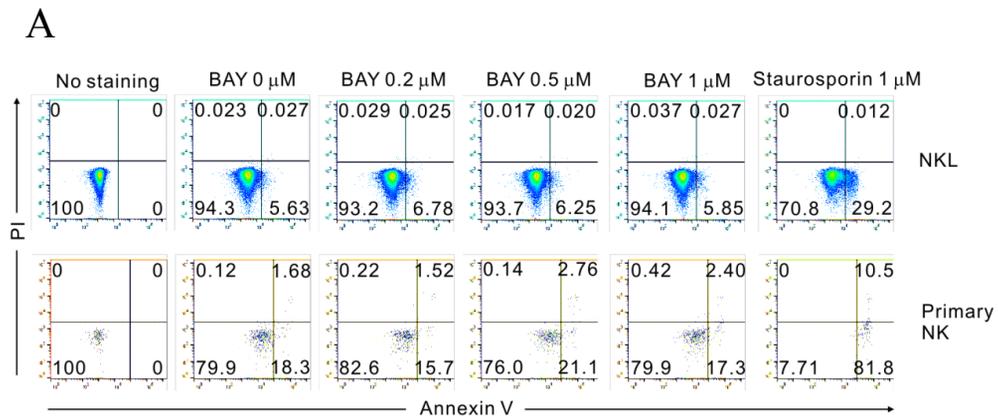


Fig. 47. Effects of BAY11-7082 on the viability of NK cells.

Rested NKL cells or primary rested NK cells after expansion were treated with BAY11-7082 or Staurosporin at the indicated dose for 9 h, then stained with Annexin-V and PI, and analyzed with flow cytometry. Representative result (A) and statistical bar charts (B) for the frequency of Annexin V⁺ cells are shown. Values represent mean ± s.d. Data are representative of three independent experiments.

III-4-3. Disparate signals converge on the NF- κ B p65 subunit

NF- κ B activation occurs primarily via IKK-dependent phosphorylation and degradation of I κ B proteins, followed by nuclear translocation of the released NF- κ B dimers. In addition, optimal NF- κ B activation relies on post-translational modifications of NF- κ B subunits, such as p65 phosphorylation, by specific protein kinases (25, 26). These emerging and additional layers of NF- κ B regulation represent an important means for crosstalk between different signaling pathways and determining context-specific transcriptional responses (27). NKG2D and 2B4 coengagement induces two distinct pathways that lead to the activation of PI3K-Akt and Vav1-dependent synergistic signaling involving PLC- γ 2 and Erk (4). Given the site-selective phosphorylation of p65 through Akt and Erk pathways (26), Vav1-dependent synergy achieved by a proximal convergence of signals from coactivation receptors may not suffice to activate NF- κ B. While stimulation of NKL cells with NKG2D or 2B4 alone induced weak p65 phosphorylation at serine 276 and 536, NKG2D and 2B4 coengagement functioned synergistically to increase their phosphorylation (Fig. 48). In support, synergistic phosphorylation of p65 was observed after stimulation with P815 cells expressing ULBP1 and CD48 (Fig. 49). To assess the dependence of such phosphorylation on Akt and Erk pathways, NKL cells were treated with an inhibitor of PI3K (LY294002) or MEK (PD98059), which block Akt or Erk activation, respectively. Notably, p65 phosphorylation at serine 276 fully depended on NKG2D and 2B4 coengagement-induced Erk activation (Fig. 50). In contrast, p65 phosphorylation at serine 536 largely depended on NKG2D-induced PI3K-Akt. The PI3K-Akt and synergistic Erk pathway appear to be mutually

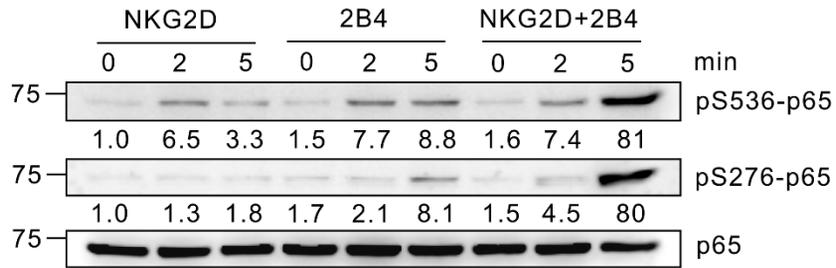


Fig. 48. NKG2D and 2B4 coengagement induces synergistic phosphorylation of p65 in NKL cells at serine 536 and 276.

Rested NKL cells were stimulated with NKG2D and/or 2B4 by receptor crosslinking for the indicated time. Cell lysates were immunoblotted for phospho-p65 at serine 536 (pS536), phospho-p65 at serine 276 (pS276), or p65. The normalized intensities of the phosphorylated p65 relative to total p65 are presented. Data are representative of at least three independent experiments.

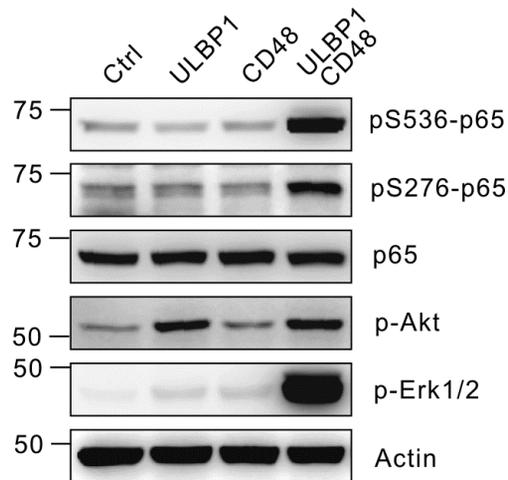


Fig. 49. Synergistic phosphorylation of p65 by NKG2D and 2B4 ligands.

Rested NKL cells were stimulated with P815 cells expressing ULBP1 and/or CD48. Cell lysates were immunoblotted with Abs specific for pS536-p65, pS276-p65, p65, p-Akt, p-Erk1/2, or actin. Data are representative of three independent experiments.

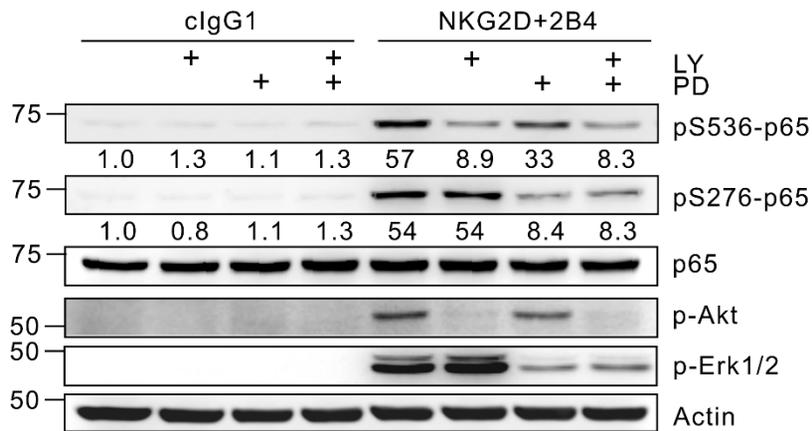


Fig. 50. Phosphorylation of p65 at serine 536 or 276 in NKL cells depends on separate Akt or Erk pathway.

Rested NKL cells were stimulated with NKG2D and 2B4 for 5 min after pretreatment with PI3K inhibitor (LY294002; 20 μ M) and/or MEK inhibitor (PD98059; 20 μ M) for 30 min. Lysates were analyzed by immunoblotting for the indicated phosphorylations of p65. Data are representative of at least three independent experiments.

independent, given the insensitivity of synergistic Erk activation to PI3K inhibitors and Akt activation to MEK inhibitors (4). The functional significance of these pathways was supported by significant impairment of IFN- γ and MIP-1 α production by an inhibitor of each pathway and further by the combined inhibition of both pathways (Fig. 51). To probe the direct role of these kinase pathways, we performed knockdown of Akt1 and/or Erk2 in NKL cells. Both Akt1 and Erk2 were required for optimal p65 phosphorylation and IFN- γ and MIP-1 α production following coactivation, complementing the results with small molecule inhibitors (Fig. 52).

We next assessed these findings using primary rested human NK cells. Similar to the results seen in NKL cells, NKG2D and 2B4 coengagement led to synergistic phosphorylation of p65 at serine 276 and 536 (Fig. 53). Moreover, we observed that phosphorylation of S276-p65 was selectively dependent on Erk pathway, while phosphorylation of S536-p65 largely relied on Akt (Fig. 54). As expected, synergistic expression of IFN- γ and TNF- α was diminished by inhibiting Akt, Erk, or both pathways (Fig. 55), correlating with reduced NF- κ B activation, as determined using reporter cells (NKL- κ B-GFP) (Fig. 56). The combined inhibition of Akt and Erk pathways demonstrated an additive effect on dampening NF- κ B activation, suggesting separate involvement of Akt and Erk pathways in NF- κ B activation.

To determine whether p65 phosphorylation is similarly induced by a different combination of coactivation receptors, primary NK cells were examined after engaging DNAM-1, 2B4, or both. DNAM-1 synergizes with 2B4 to trigger effector functions (28). Notably, crosslinking DNAM-1 induced Akt phosphorylation, as did NKG2D, and 2B4 coengagement induced a synergistic

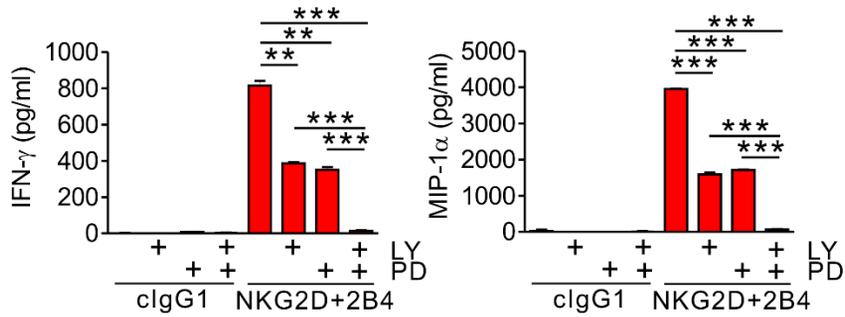
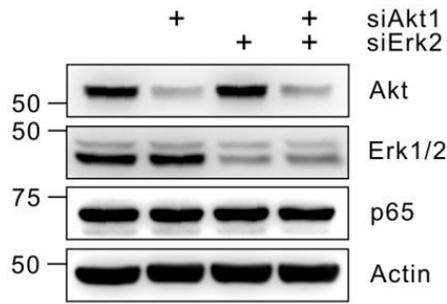


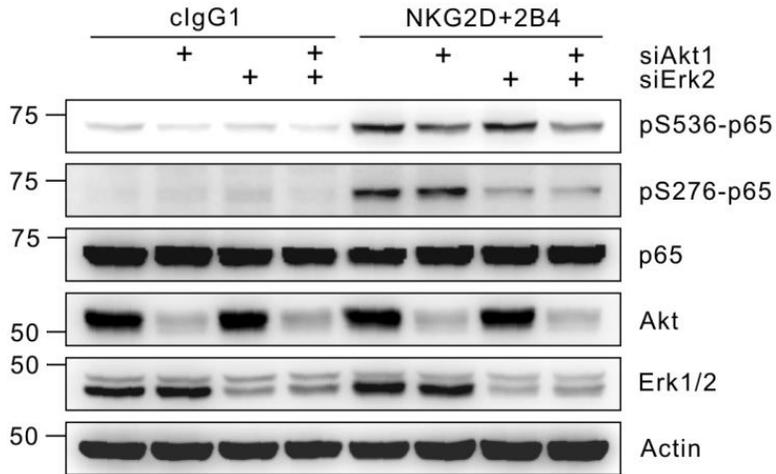
Fig. 51. Akt and Erk pathway are required for NK cells cytokines secretion.

Cytokine release assays with rested NKL cells after pretreatment with 20 μ M LY294002 and/or 20 μ M PD98059 for 30 min and then stimulation with NKG2D and 2B4 for 12 h in the presence of the inhibitor. Thereafter, IFN- γ (left) and MIP-1 α (right) in the supernatants were measured by ELISA. Values represent mean \pm s.d. ** $P < 0.01$; *** $P < 0.001$ (two-sided Student's t -test). Data are representative of at least three independent experiments.

A



B



C

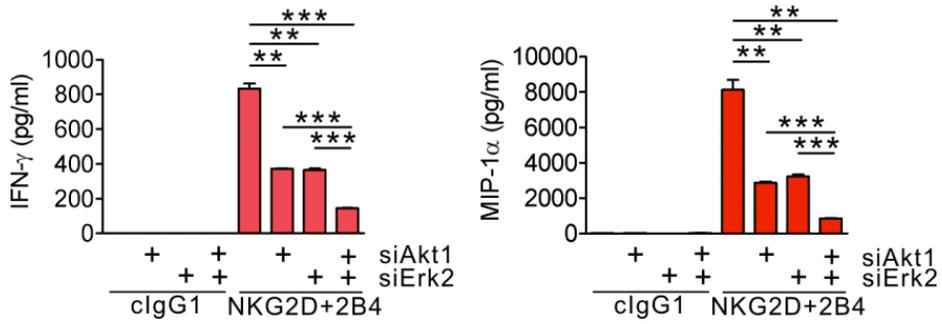


Fig. 52. Requirement of Akt1 and Erk2 for optimal p65 phosphorylation and IFN- γ and MIP-1 α production following coactivation.

(A) NKL cells were transfected with control siRNA or siRNAs specific for Akt1 and/or Erk2. Lysates were immunoblotted for Akt, Erk1/2, p65, or actin.

(B) Rested NKL cells transfected with control siRNA or siRNAs specific for Akt1 and/or Erk2 were stimulated with NKG2D and 2B4 by receptor crosslinking. Lysates were immunoblotted for pS536-p65, pS276-p65, p65, Akt, Erk1/2, or actin.

(C) Cytokine release assays with rested NKL cells after transfection with control siRNA or siRNAs specific for Akt1 and/or Erk2 and then stimulation with NKG2D and 2B4 for 8 h. Thereafter, IFN- γ (left) and MIP-1 α (right) in the supernatants were measured by ELISA. Values represent mean \pm s.d. ** P <0.01; *** P <0.001 (two-sided Student's t -test). Data are representative of three independent experiments.

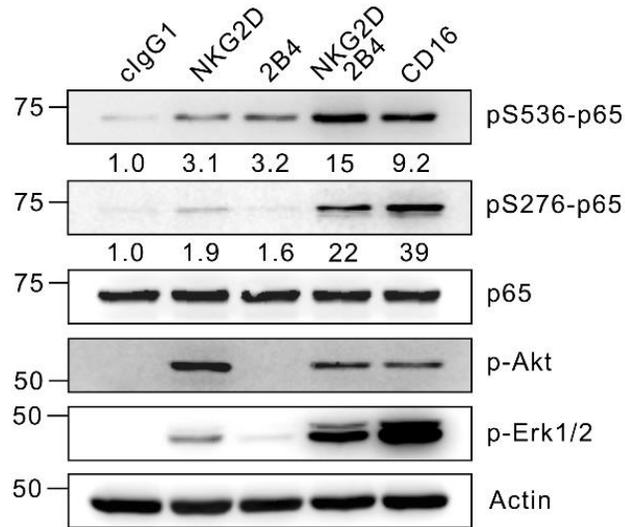


Fig. 53. NKG2D and 2B4 coengagement induces synergistic phosphorylation of p65 in primary NK cells at serine 536 and 276.

Primary rested NK cells after expansion were stimulated with the indicated receptors by receptor crosslinking for 5 min. Lysates were analyzed by immunoblotting as in Fig. 50. Data are representative of three independent experiments.

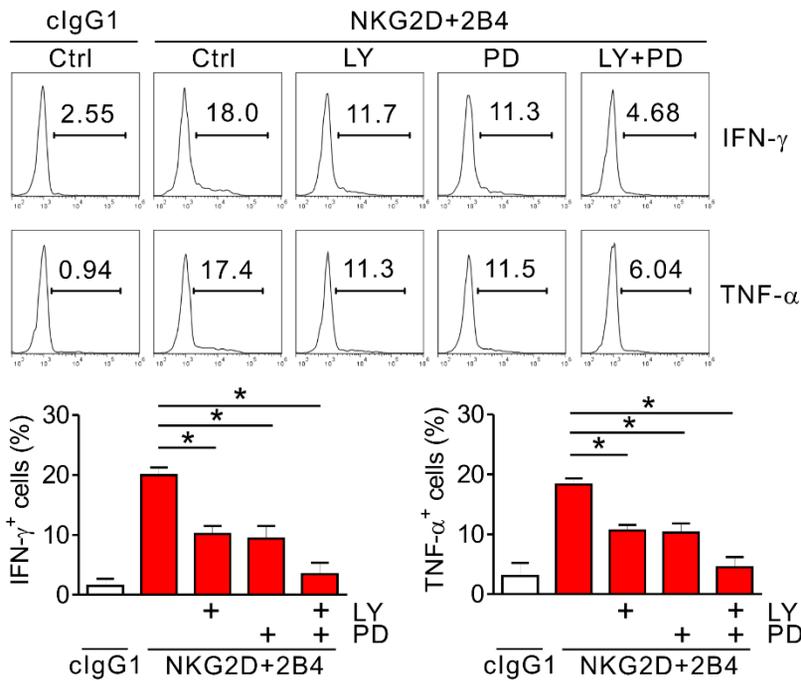


Fig. 55. Requirement of Akt and Erk pathways for IFN- γ and TNF- α production following NKG2D and 2B4 coactivation.

Frequency of NK cells that displayed IFN- γ or TNF- α expression after pretreatment of PBMCs with 20 μ M LY294002 and/or 20 μ M PD98059 for 1 h and then stimulation with P815 target cells as indicated in the presence of the inhibitor. After incubation for 6 h, cells were stained and analyzed by flow cytometry. Representative result (top) and statistical bar charts (bottom) from three experiments are shown. Values represent mean \pm s.e.m. * P < 0.05 (two-sided Student's t -test). Data are representative of three independent experiments.

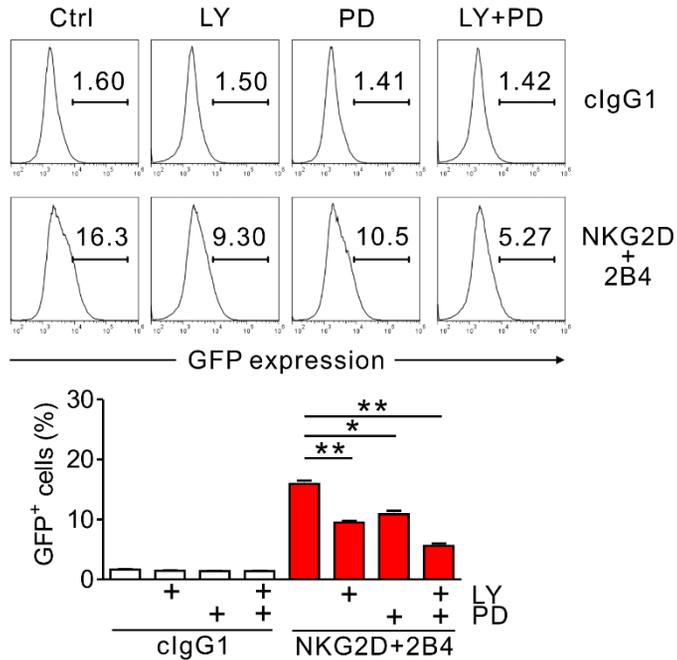
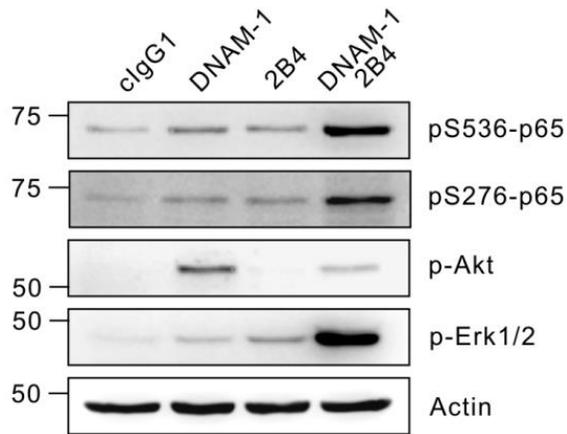


Fig. 56. Requirement of Akt and Erk pathways for transcriptional activity of NF- κ B following NKG2D and 2B4 coactivation.

Rested NKL- κ B-GFP cells were pretreated with 20 μ M LY294002 and/or 20 μ M PD98059 for 1 h and then stimulated with plate-immobilized NKG2D and 2B4 for 6 h. GFP expression in the reporter NKL cells was analyzed by flow cytometry, and representative result (top) and statistical bar charts (bottom) are shown. Values represent mean \pm s.d. ** P < 0.01 (two-sided Student's t -test). Data are representative of at least three independent experiments.

phosphorylation of Erk, an apparent dual phosphorylation of p65, and synergistic NF- κ B activation (Fig. 57). These suggest that the basis for NF- κ B activation is shared between DNAM-1 and NKG2D. In contrast, crosslinking CD16, which mediates antibody-dependent cellular cytotoxicity and is sufficient for cytokine production, induced dual phosphorylation of p65 in addition to Akt and Erk phosphorylation (Fig. 53). These suggest distinct regulation of p65 phosphorylation by coactivation receptors for natural cytotoxicity that are incapable of activating alone. Thus, coengagement of 2B4 with NKG2D, or DNAM-1, was required to achieve signaling competence for p65 phosphorylation and NF- κ B activation.

A



B

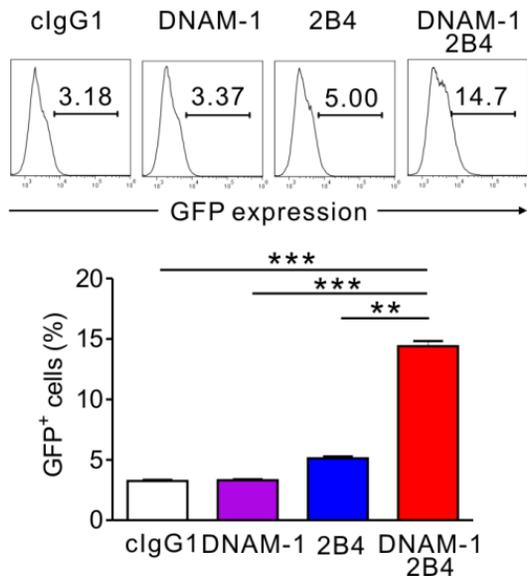


Fig. 57. DNAM-1 synergizes with 2B4 to induce competent p65 phosphorylation and NF- κ B activation.

(A) Primary rested NK cells after expansion were stimulated with DNAM-1 and/or 2B4 for 2 min. Thereafter, the lysates were immunoblotted with Abs to pS536-p65, pS276-p65, p-Akt, p-Erk1/2, or actin.

(B) Rested NKL- κ B-GFP cells were stimulated with plate-immobilized mAbs specific for DNAM-1 and/or 2B4 for 6 h. GFP expression in NKL- κ B-GFP cells was analyzed by flow cytometry, and representative result (top) and statistical bar charts (bottom) are shown. Values represent mean \pm s.d. $**P < 0.01$; $***P < 0.001$ (two-sided Student's *t*-test). Data are representative of three independent experiments.

III-4-4. Signal amplification is insufficient for NF- κ B activation

The dependence of NK cell activation on synergistic signals is, in part, relieved after c-Cbl knockdown (4). Thus, we examined whether depleting c-Cbl enables NKG2D or 2B4 to bypass the requirement for synergistic signals to induce p65 phosphorylation and NF- κ B activation. c-Cbl knockdown slightly enhanced p65 phosphorylation at serine 536 and 276 in response to NKG2D or 2B4 alone (Fig. 58). However, it remained lower than p65 phosphorylation by their synergistic coactivation. In contrast, c-Cbl knockdown substantially enhanced p65 phosphorylation at both serines following synergistic coactivation.

Given the role of Akt and Erk in the phosphorylation of S536-p65 and S276-p65, we next assessed the effects of c-Cbl knockdown on Akt and Erk phosphorylation. While Akt phosphorylation induced by NKG2D was markedly augmented by c-Cbl knockdown, it was undetectable following 2B4 stimulation even after c-Cbl knockdown (Fig. 58). In comparison, c-Cbl knockdown caused a small increase in Erk phosphorylation by NKG2D or 2B4 alone, but an apparent increase by their synergistic coactivation. These results suggest that c-Cbl knockdown may amplify signal input, but not trigger disparate signals for NF- κ B activation.

The extent of pS276-p65 induced by NKG2D, 2B4, or both correlated with the level of Erk phosphorylation (Fig. 58). In contrast, the correlation between pS536-p65 and Akt phosphorylation was observed following NKG2D and 2B4 coactivation, but not NKG2D alone, although there was comparable Akt phosphorylation. This suggests a checkpoint that restrains pS536-p65 by Akt

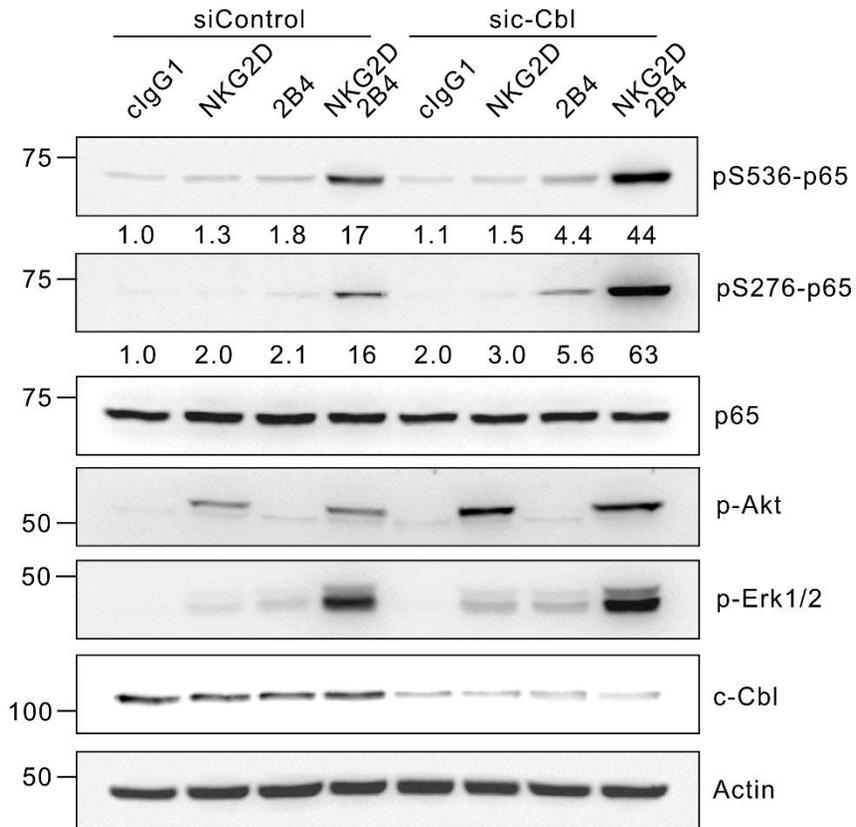


Fig. 58. c-Cbl depletion augments but is not sufficient for NF- κ B phosphorylation.

Rested NKL cells transfected with control siRNA or c-Cbl-specific siRNA were stimulated through the indicated receptors for 5 min. Cell lysates were immunoblotted for phospho-p65 at serine 536 (pS536), phospho-p65 at serine 276 (pS276), p65, phospho-Akt at serine 473 (pS473), phospho-Erk1 and 2, c-Cbl, or actin. The normalized intensities of the phosphorylated p65 relative to p65 are presented. Data are representative of three independent experiments.

pathway, which could be overcome by synergistic coactivation. In support, NF- κ B activation and production of IFN- γ and MIP-1 α by NKG2D and 2B4 coactivation but not by a single receptor was markedly enhanced after c-Cbl knockdown (Fig. 59,60). Together, signal amplification from individual receptor could not compensate for the lack of complementary signals from its partner receptor for NF- κ B activation.

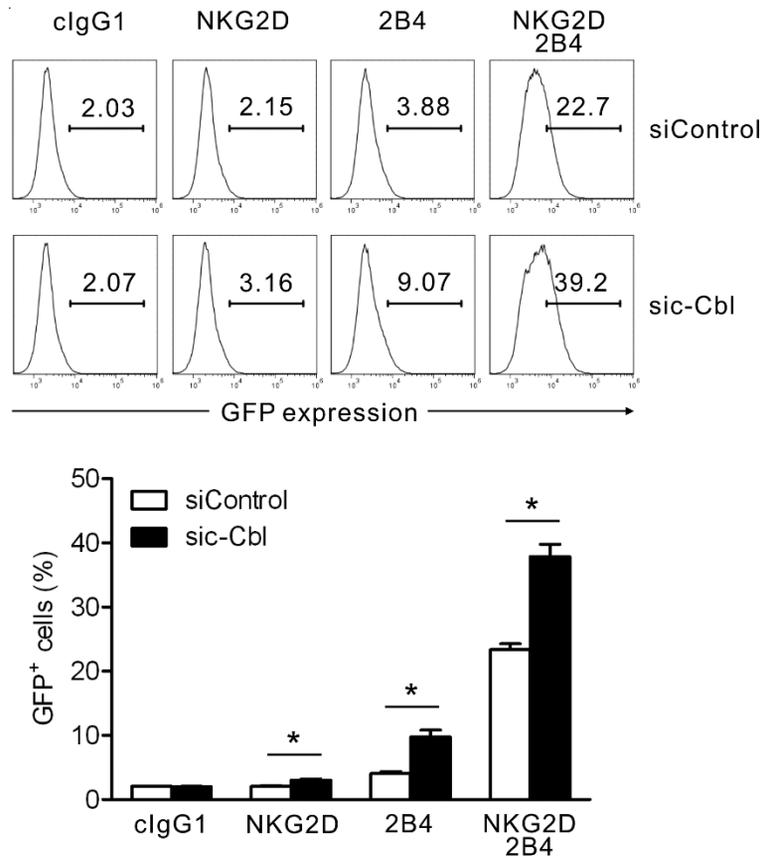


Fig. 59. c-Cbl depletion augments but is not sufficient for transcriptional activity of NF- κ B.

Rested NKL- κ B-GFP cells transfected with control siRNA or c-Cbl-specific siRNA were stimulated with plate-immobilized mAbs specific for NKG2D and/or 2B4 for 6 h. GFP expression in NKL- κ B-GFP cells was analyzed by flow cytometry, and representative result (top) and statistical bar charts (down) are shown. Values represent mean \pm s.d. * $P < 0.05$ (two-sided Student's t -test). Data are representative of at least three independent experiments.

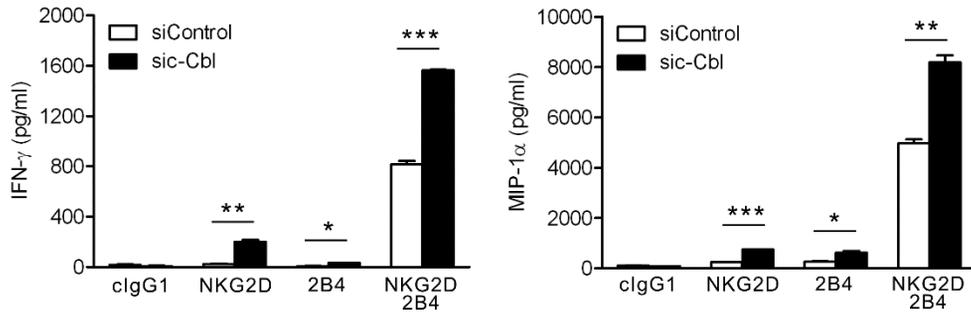


Fig. 60. c-Cbl depletion augments but is not sufficient for cytokines secretion by NKL cells.

Cytokine release assays with rested NKL cells transfected with control siRNA or c-Cbl-specific siRNA and stimulated with NKG2D and/or 2B4 for 8 h. IFN- γ and MIP-1 α in the supernatants were measured by ELISA. Values represent mean \pm s.d. * P < 0.05; ** P < 0.01; *** P < 0.001 (two-sided Student's t -test). Data are representative of at least three independent experiments.

III-4-5. Defective NF- κ B activation in XLP1 NK cells by NKG2D and 2B4

We next assessed whether NF- κ B activation requires cooperation between coactivation receptors in pathophysiological contexts. XLP1 is characterized by severe immunodeficiency resulting from mutations in the *SH2D1A* gene encoding the SAP protein (29-31). Given the requirement of SAP for 2B4-dependent NK cell activation (32, 33), we hypothesized that NK cells from XLP1 patients would demonstrate defects in NF- κ B activation and NK cell responses following coactivation. We first tested the requirement of SAP for NK cell activation during NKG2D and 2B4 synergy by performing siRNA-mediated knockdown of SAP in NKL cells (Fig. 61). The synergistic increases in Ca²⁺ mobilization, NF- κ B activation, and IFN- γ and MIP-1 α release were markedly diminished by SAP knockdown (Fig. 62,63,64). Likewise, SAP knockdown markedly reduced the synergistic increase in p65 phosphorylation and the proportion of NK cells expressing IFN- γ in primary NK cells following NKG2D and 2B4 coactivation (Fig. 65A, B). The small increase in IFN- γ expression by ligating 2B4 but not NKG2D alone was also selectively decreased by SAP knockdown.

Given these promising results, NK cells from XLP1 patients were used to study the dependence of synergistic coactivation on SAP expression. Among the four XLP1 patients examined, three patients harbored macrodeletions in the *SH2D1A* gene that resulted in complete loss of SAP expression, and one patient harbored a missense mutation that reduced SAP expression, as assessed by Western blot analysis (Fig. 66A, B). To probe the functional defects in XLP1 NK cells, we measured target



Fig. 61. SAP-specific knockdown in NKL cells.

NKL cells were transfected with 300 pmoles of control siRNA or siRNA specific for SAP. After 24 h, the cells were rested for another 24 h, and the lysates were immunoblotted for SAP or actin. Data are representative of at least three independent experiments.

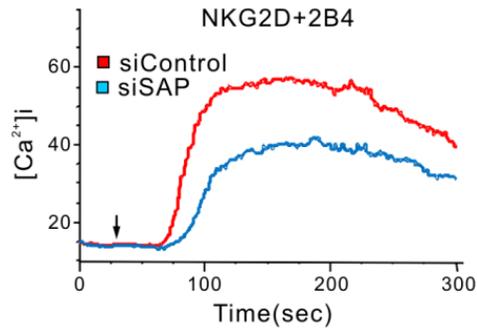


Fig. 62. SAP is required for Ca^{2+} mobilization by NKG2D and 2B4 coactivation.

Ca^{2+} mobilization in rested NKL cells transfected with control siRNA or SAP-specific siRNA. NKL cells were stimulated through NKG2D and 2B4 (indicated by the arrow) after the measurement of baseline Ca^{2+} concentrations for 30 s. Changes in fluorescence are shown as a function of time. Data are representative of at least three independent experiments.

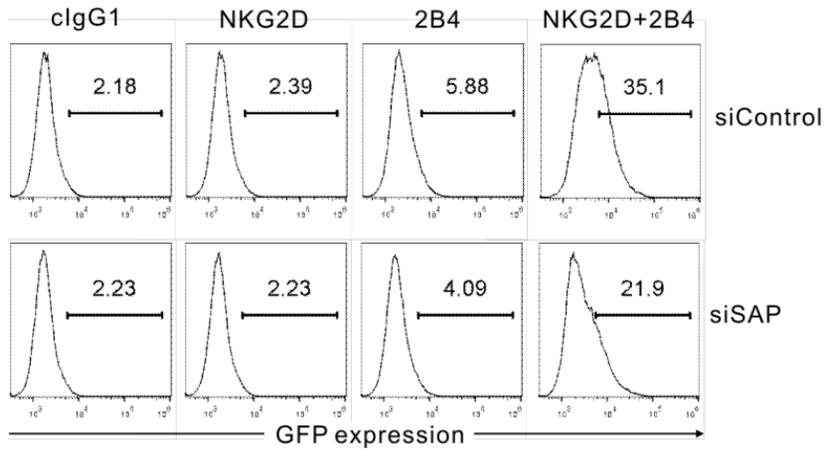


Fig. 63. SAP is required for NF- κ B transcriptional activity by NKG2D and 2B4 coactivation.

Rested NKL- κ B-GFP cells transfected with control siRNA or SAP-specific siRNA were stimulated with plate-immobilized mAbs specific for NKG2D and/or 2B4 for 6 h. GFP expression in the reporter NKL cells was analyzed using flow cytometry. Data are representative of at least three independent experiments.

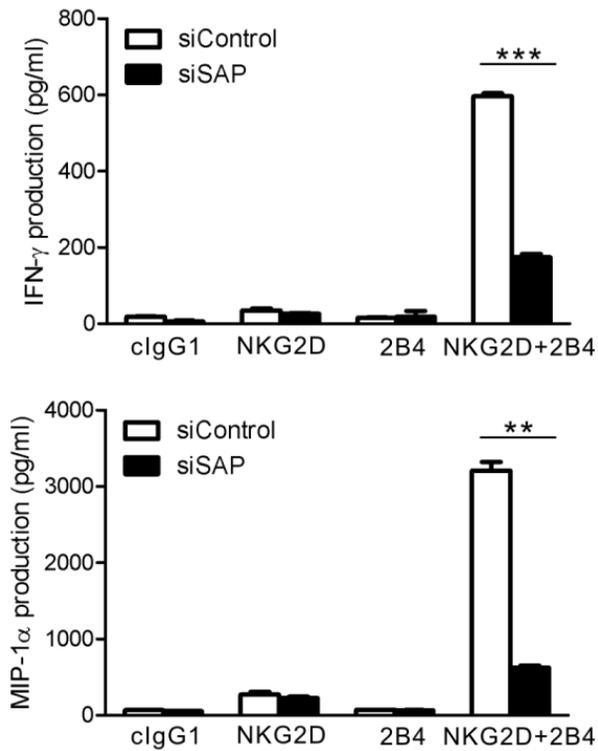
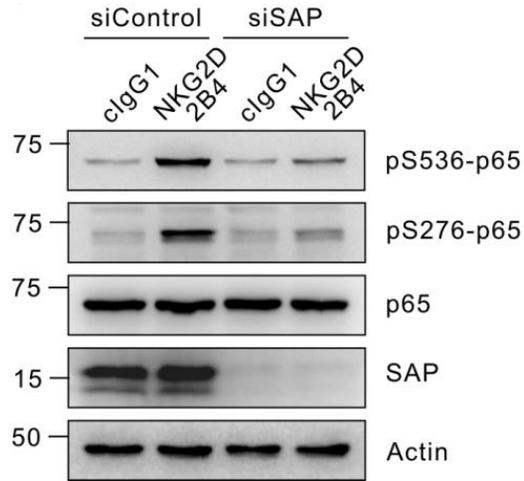


Fig. 64. SAP is required for cytokines secretion by NKG2D and 2B4 coactivation.

Cytokine release assays with rested NKL cells transfected with control siRNA or SAP-specific siRNA and stimulated with NKG2D and/or 2B4. After incubation for 12 h, IFN- γ (top) and MIP-1 α (bottom) in the supernatants were measured by ELISA. Values represent the mean \pm s.d. $**P < 0.01$; $***P < 0.001$ (two-sided Student's *t*-test). Data are representative of three independent experiments.

A



B

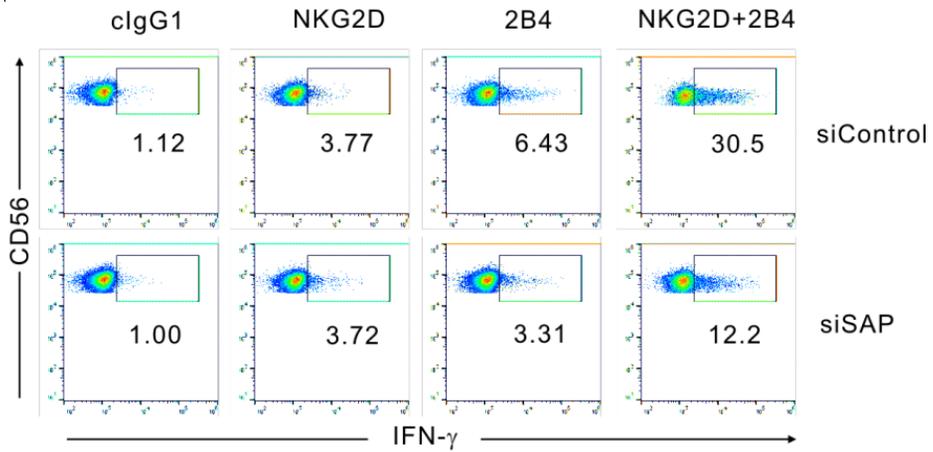


Fig. 65. SAP is required for p65 phosphorylation and IFN- γ expression in primary NK cells via NKG2D and 2B4 coactivation.

(A) Primary rested NK cells after expansion that were transfected with control siRNA or SAP-specific siRNA were stimulated with NKG2D and 2B4 by receptor crosslinking. Lysates were immunoblotted for pS536-p65, pS276-p65, p65, SAP, or actin.

(B) Primary rested NK cells after expansion that were transfected with control siRNA or SAP-specific siRNA were stimulated with P815 target cells as indicated. After incubation for 6 h, cells were stained, and frequency of NK cells that displayed intracellular IFN- γ expression was analyzed by flow cytometry. Data are representative of three independent experiments.

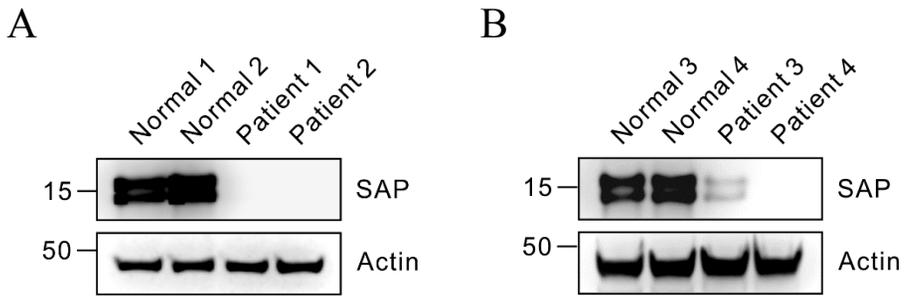


Fig. 66. SAP deficiency in XLP1 NK cells.

(A) Total lysates of primary expanded NK cells from normal (normal 1 and 2) or XLP1 patient (patient 1 and 2) donors were immunoblotted for SAP and actin.

(B) Total lysates of primary expanded NK cells from normal (normal 3 and 4) or XLP1 patient (patient 3 and 4) donors were immunoblotted for SAP and actin.

cell-induced degranulation, as determined by cell surface expression of CD107a (28). Stimulation with K562 cell line induced strong degranulation of both normal and XLP1 NK cells (Fig. 67A, B). In contrast, XLP1 NK cells were severely impaired in their ability to degranulate against EBV-immortalized B-lymphoblastoid cell line 721.221 (referred to as 221), an observation compatible with the defective killing of EBV-infected B cells by XLP1 NK cells (32).

Next, we tested whether SAP is required for NF- κ B activation and NK cell responses in XLP1 NK cells. Notably, the apparent dual phosphorylation of p65 at serine 536 and 276 following NKG2D and 2B4 coactivation seen in normal NK cells was significantly diminished in XLP1 NK cells (Fig. 68). The synergistic phosphorylation of Erk following coactivation was also diminished in XLP1 NK cells, whereas Akt phosphorylation induced by NKG2D appeared to be unaffected. In contrast, dual phosphorylation of p65 by CD16 ligation was comparable in normal and XLP1 NK cells (Fig. 68). Moreover, NF- κ B activation in XLP1 NK cells was measured by nuclear translocation of p65 in individual NK-target cell conjugates. A significant defect in nuclear translocation of p65 following coactivation was detected in XLP1 NK cells relative to normal NK cells (Fig. 69). Corroborating these findings, the proportion of NK cells expressing IFN- γ was severely decreased in XLP1 NK cells following NKG2D and 2B4 coactivation, but not CD16 (Fig. 70A, B). Most strikingly, the small increase in IFN- γ expression by 2B4 ligation was selectively defective in XLP1 NK cells, consistent with a critical role of SAP in 2B4-mediated signaling. A similar but less severe defect was observed when degranulation was induced through 2B4 alone and in combination with NKG2D, but not through CD16 (Fig. 71A, B). These results did not appear to be associated with defective expression

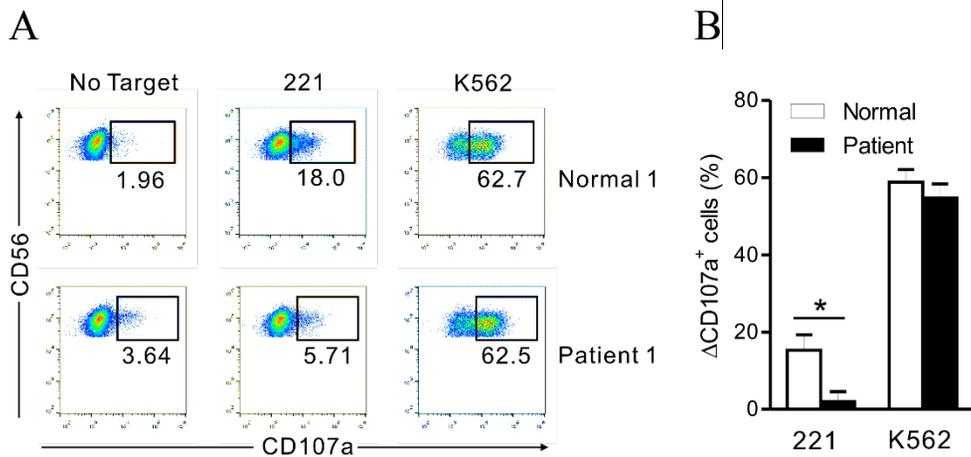


Fig. 67. XLP1 NK cells have defects in degranulation in response to 221 target cells.

(A and B) Primary rested NK cells after expansion from normal or XLP1 patient donors were mixed with 221 or K562 cells in the presence of fluorochrome-conjugated anti-CD107a mAb for degranulation assay. After incubation for 2 h, cells were stained with fluorochrome-conjugated mAb to CD56, and the level of CD56⁺CD107a⁺ NK cells was measured using flow cytometry. (A) Representative result is shown. (B) Percent increase of CD107a⁺ NK cells obtained from normal or XLP1 donors after stimulation with target cells relative to CD107a⁺ NK cells without target cells (Δ CD107a⁺ cells). Values represent the mean \pm s.e.m. * P < 0.05 (two-sided Student's t -test).

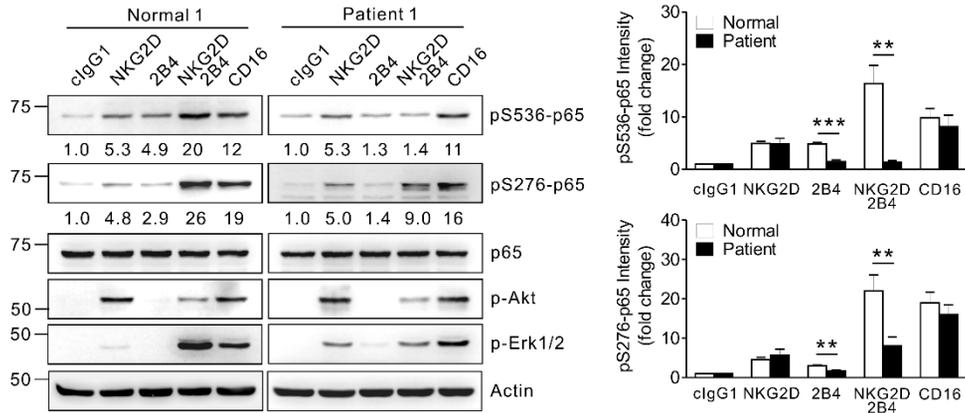


Fig. 68. XLP1 NK cells have defects in phosphorylation of p65 at serine 536 and 276 and Erk by NKG2D and 2B4 coactivation.

Primary rested NK cells after expansion from normal or XLP1 patient donors were stimulated with the indicated receptors for 5 min. Lysates were immunoblotted for the indicated phosphorylations of p65. The normalized intensities of the phosphorylated p65 relative to p65 are presented. Representative result (left) and statistical bar charts (right) are shown. Values represent mean \pm s.e.m. $**P < 0.01$; $***P < 0.001$ (two-sided Student's *t*-test). Statistical bar charts show pooled data from three different donors.

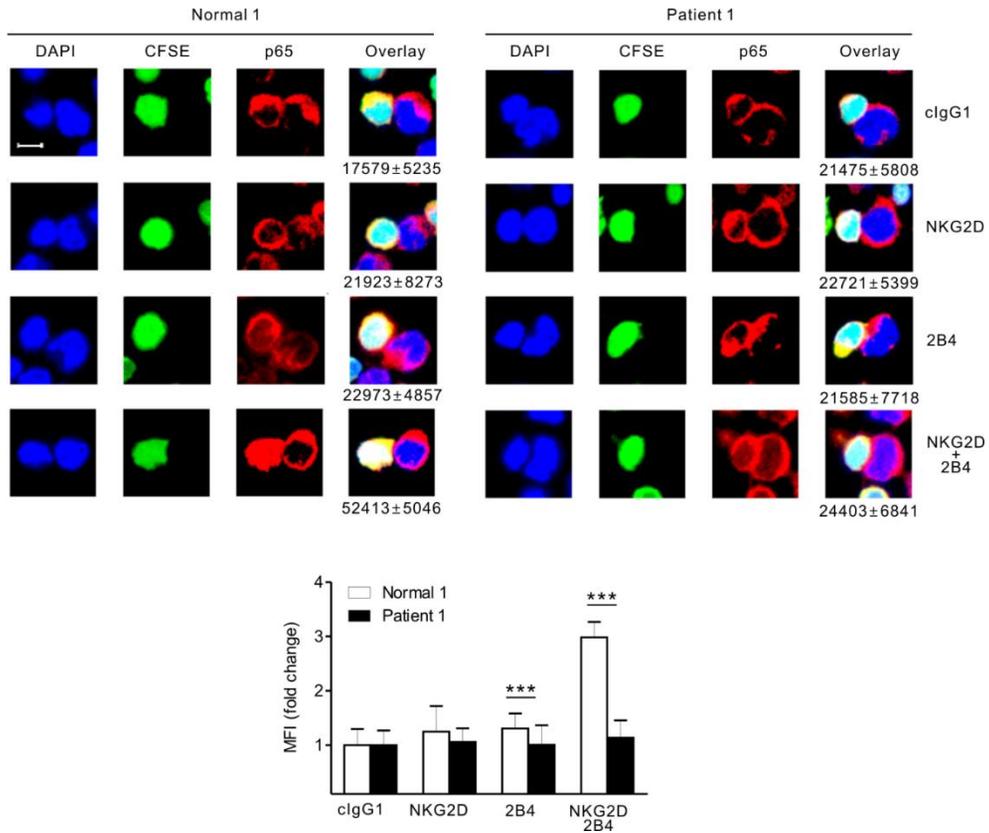


Fig. 69. XLP1 NK cells have defects in p65 nuclear translocation in response to NKG2D and 2B4 coactivation.

Representative confocal images (top) of conjugates between CFSE (green)-loaded primary rested NK cells from normal or XLP1 donor and P815 target cells as indicated. Conjugates were fixed, permeabilized, and stained with DAPI (blue) and mAb to p65, anti-mouse IgG-Biotin followed with Alexa Fluor 647 (red)-Streptavidin. Mean nuclear MFI ± s.d. of p65 in ≥ 30 NK-target cell conjugates are shown beneath the overlay image. Statistical bar charts (bottom) for MFI of p65 in the nucleus are represented as fold change. Values represent mean ± s.d. *** $P < 0.001$ (two-sided Student's *t*-test). Scale bar, 5 μm .

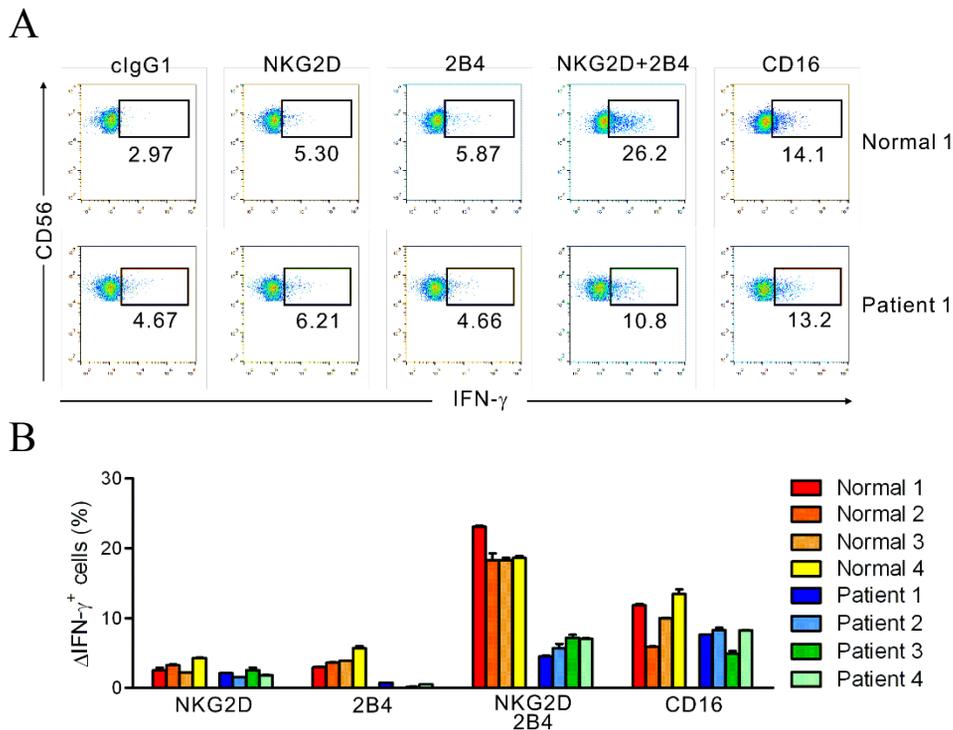


Fig. 70. XLP1 NK cells have defects in IFN- γ production following NKG2D and 2B4 coactivation.

(A and B) Primary rested NK cells after expansion from normal or XLP1 patient donors were mixed with P815 target cells as indicated. After incubation for 6 h, cells were stained with fluorochrome-conjugated mAb to CD56 and analyzed by flow cytometry after intracellular staining of IFN- γ . (A) Representative result is shown. (B) Percent increase of IFN- γ + NK cells from individual normal or XLP1 patient donors after stimulation with the indicated receptors relative to IFN- γ + NK cells without stimulation (Δ IFN- γ + cells) is presented. Values represent mean \pm s.d.

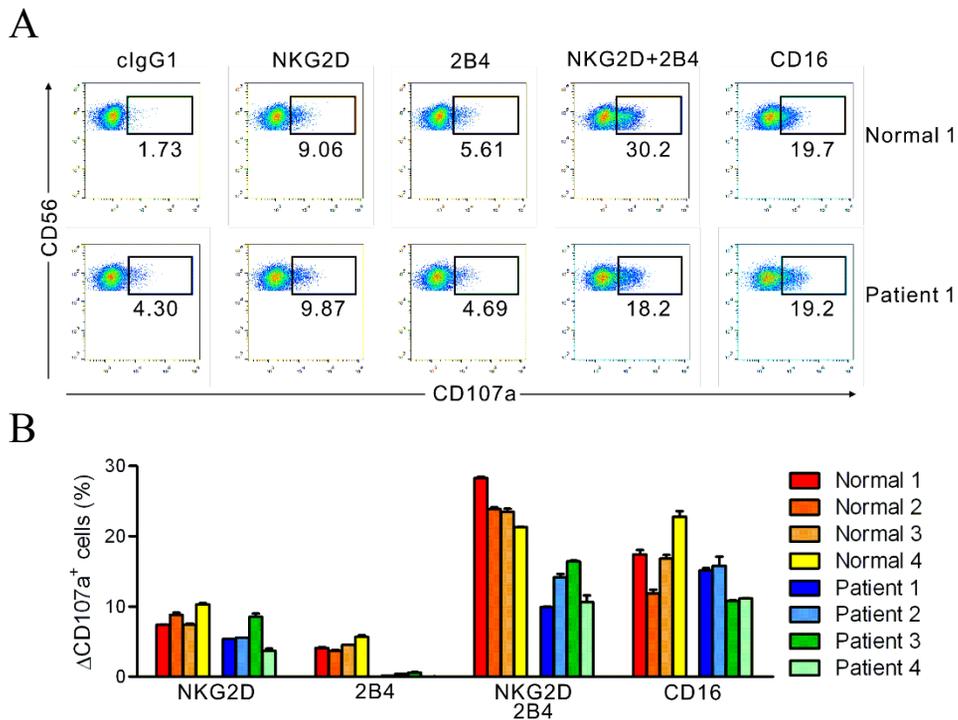


Fig. 71. XLP1 NK cells have defects in degranulation following NKG2D and 2B4 coactivation.

(A and B) Primary rested NK cells after expansion from normal or XLP1 donors were mixed with P815 target cells as indicated in the presence of fluorochrome-conjugated anti-CD107a mAb. After incubation for 2 h, cells were analyzed using flow cytometry as described in Fig. 67A. (A) Representative result is shown. (B) Percent increase of CD107a⁺ NK cells obtained from individual normal or XLP1 donors after stimulation with the indicated receptors relative to CD107a⁺ NK cells without stimulation (Δ CD107a⁺ cells). Values represent mean \pm s.d.

of coactivation receptors in XLP1 NK cells, given comparable expression of various activating receptors, including NKG2D, 2B4, and CD16 (Fig. 72). Instead, our results suggest that the functional deficiencies in XLP1 NK cells by NKG2D and 2B4 coactivation are most likely due to selective defects in 2B4-associated synergistic signaling to Erk, which is important for optimal NF- κ B activation and effector functions.

Enhanced Vav1 signaling during NKG2D and 2B4 coactivation is required to overcome inhibition by c-Cbl and deliver synergistic signals for NK cell activation (4). Thus, we tested whether aberrant Vav1 regulation is associated with such defects seen in XLP1 NK cells. As reported, Vav1 phosphorylation was induced in normal NK cells by engaging NKG2D or 2B4, and was additive after coengagement (Fig. 73). Notably, Vav1 phosphorylation was impaired in XLP1 NK cells following 2B4 stimulation, but normal after NKG2D stimulation. Furthermore, Vav1 phosphorylation after co-engaging NKG2D and 2B4 was defective in XLP1 NK cells, similar to the level of phosphorylation induced by NKG2D alone (Fig. 73). Collectively, SAP deficiency could cause a defect in 2B4, but not NKG2D, signaling at the level of Vav1 and thereby impair the synergistic activation of Erk and NF- κ B following coactivation.

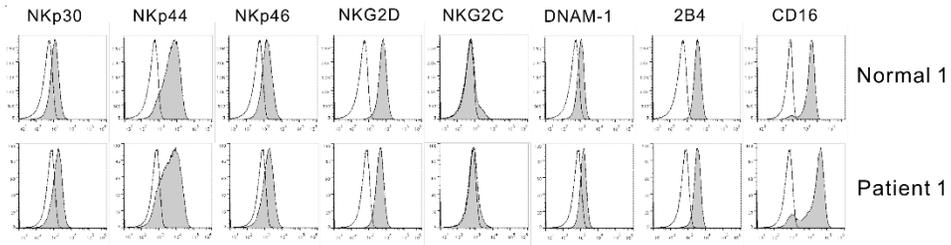


Fig. 72. Expression profile of activating receptors in normal and XLP1 NK cells.

Representative FACS profiles showing the expression levels of the NKp30, NKp44, NKp46, NKG2D, NKG2C, DNAM-1, 2B4, and CD16 receptors (shaded histogram) on primary expanded NK cells obtained from normal or XLP1 donors. Isotype control staining is shown as the solid lines.

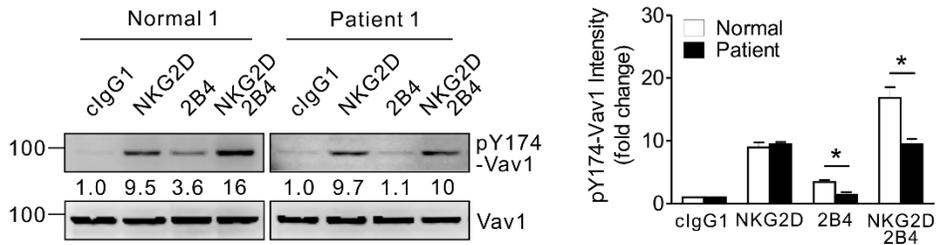


Fig. 73. XLP1 NK cells have defects in Vav1 phosphorylation following NKG2D and 2B4 coactivation.

Primary rested NK cells after expansion from normal or XLP1 patient donors were treated as in Fig. 68 to stimulate NKG2D and/or 2B4 for 2 min. Lysates were immunoblotted with anti-pY174-Vav1 Ab and reprobbed for Vav1. The normalized intensities of the phosphorylated Vav1 relative to total Vav1 are presented. Representative result (left) and statistical bar charts for pooled data from three different donors (right) are shown. Values represent mean \pm s.e.m. $*P < 0.05$ (two-sided Student's *t*-test).

III-4-6. Vav1 is required for the synergistic activation of NF- κ B

To ensure that Vav1 is required for synergistic NF- κ B activation, we tested whether NF- κ B activation following coactivation is susceptible to Vav1 inhibition. Inhibitory signaling through CD94-NKG2A can override Vav1-dependent activation of NK cells (4). The synergistic phosphorylation of p65 and Erk and the combined phosphorylation of Vav1 induced by NKG2D and 2B4 coactivation were all abrogated by co-crosslinking CD94-NKG2A on NKL cells (Fig. 74). Moreover, NKG2D-dependent phosphorylation of Akt, required for pS536-p65, was also abrogated by the same inhibition. Accordingly, the synergistic increase in transcriptional activity of NF- κ B was markedly impaired by co-crosslinking CD94-NKG2A (Fig. 75). These results suggest the suppression of NF- κ B activation as a mechanism underlying the inhibitory function of CD94-NKG2A. To ascertain the direct involvement of Vav1 in NF- κ B activation, we performed siRNA-mediated knockdown of Vav1. Similarly, Vav1 knockdown abrogated the phosphorylation of p65, along with that of Akt and Erk, and in turn transcriptional activity of NF- κ B following coactivation (Fig. 76,77). Collectively, our results suggest that NF- κ B activation in NK cells by NKG2D and 2B4 coactivation is under the control of Vav1 and dominantly inhibited by CD94-NKG2A.

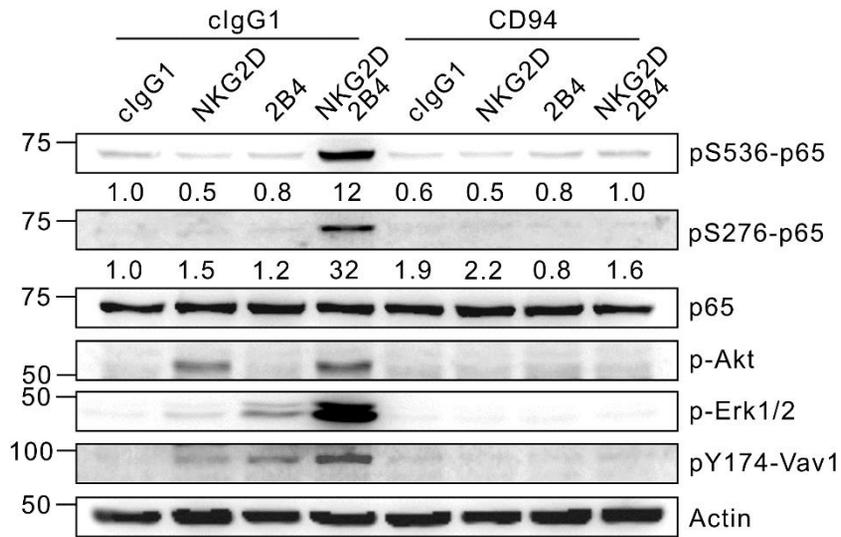


Fig. 74. Synergistic phosphorylation of p65 is impaired by co-crosslinking CD94-NKG2A.

Rested NKL cells were stimulated with NKG2D and/or 2B4 in combination with or without CD94 engagement by receptor crosslinking. Lysates were immunoblotted for pS536-p65, pS276-p65, p65, p-Akt, p-Erk1/2, pY174-Vav1, or actin. The normalized intensities of the phosphorylated p65 relative to p65 are presented. Data are representative of at least three independent experiments.

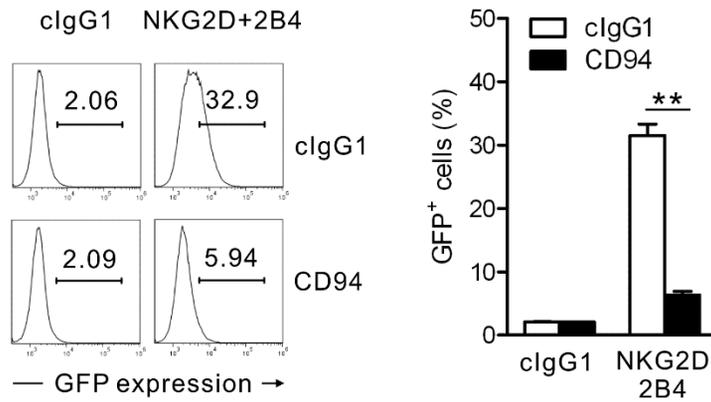


Fig. 75. Synergistic increase of NF- κ B transcriptional activity is impaired by co-crosslinking CD94-NKG2A.

Rested NKL- κ B-GFP cells were stimulated with plate-immobilized NKG2D and 2B4 in combination with or without CD94 for 6 h. GFP expression in the reporter NKL cells was analyzed by flow cytometry, and representative result (left) and statistical bar charts (right) are shown. Values represent mean \pm s.d. ****** $P < 0.01$ (two-sided Student's t -test). Data are representative of at least three independent

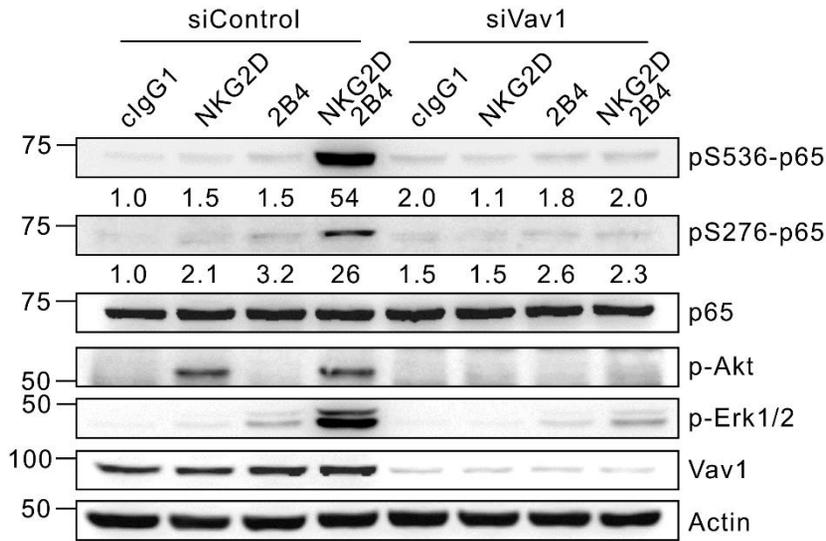


Fig. 76. Synergistic phosphorylation of p65 is impaired by Vav1 knockdown.

Rested NKL cells transfected with control siRNA or Vav1-specific siRNA were stimulated with NKG2D and/or 2B4 by receptor crosslinking. Lysates were immunoblotted for pS536-p65, pS276-p65, p65, p-Akt, p-Erk1/2, Vav1, or actin. The normalized intensities of the phosphorylated p65 relative to p65 are presented. Data are representative of at least three independent experiments.

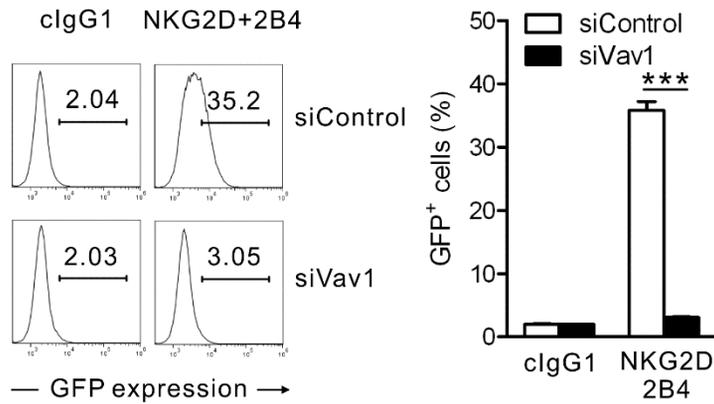


Fig. 77. Synergistic increase of NF- κ B transcriptional activity is impaired by Vav1 knockdown.

Rested NKL- κ B-GFP cells transfected with control siRNA or Vav1-specific siRNA were stimulated with plate-immobilized NKG2D and 2B4 for 6 h. GFP expression in the reporter NKL cells was analyzed by flow cytometry, and representative result (left) and statistical bar charts (right) are shown. Values represent mean \pm s.d. *** $P < 0.001$ (two-sided Student's t -test). Data are representative of at least three independent experiments.

III-5. Discussion

Here we offer a new perspective on the regulation of NF- κ B activation in NK cells during target cell recognition. NF- κ B activation via non-ITAM-associated receptors (e.g., NKG2D, 2B4, DNAM-1) relies on coordinated engagement of coactivation receptors, which together provide complementary and independent signals leading to optimal Vav1 and p65 phosphorylation (Fig. 8). The importance of such regulation between Vav1 and p65 in NF- κ B activation was supported by signaling and functional defects centered on Vav1 in XLP1 NK cells following coactivation.

A prevailing view of the mechanism underlying synergistic coactivation involves signal integration by receptor-proximal adaptor proteins that promote downstream signaling events for cytokine production and target cell killing (1). Supporting this, it was shown that signals from synergizing receptors converge on the adaptor protein SH2 domain-containing leukocyte phosphoprotein of 76 kD (SLP-76) through site-selective phosphorylation of two tyrosines in SLP-76 (34). These two phosphotyrosines enable simultaneous binding of Vav1-Nck protein complex to both tyrosines on SLP-76, which leads to Vav1-dependent synergistic signals. Vav1 is an essential component for synergistic coactivation among combinations of NKG2D, 2B4, and DNAM-1⁴. Thus, the dependence of synergistic coactivation on the regulated interaction between SLP-76 and Vav1 may represent a checkpoint in NK cell activation that ensures proper specificity of NK cell responses. Supporting this notion, the defective phosphorylation of Vav1 in XLP1 NK cells, and

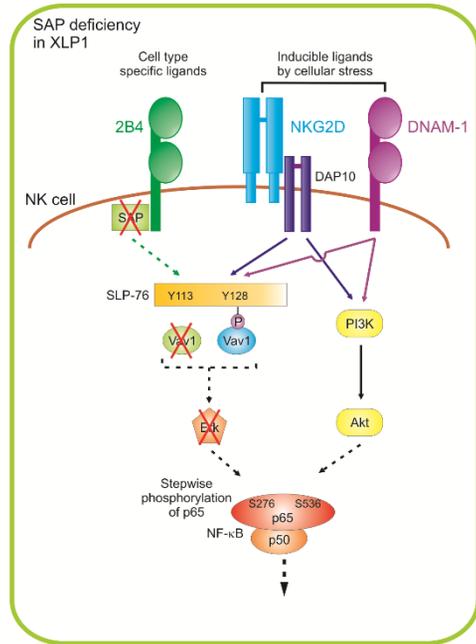
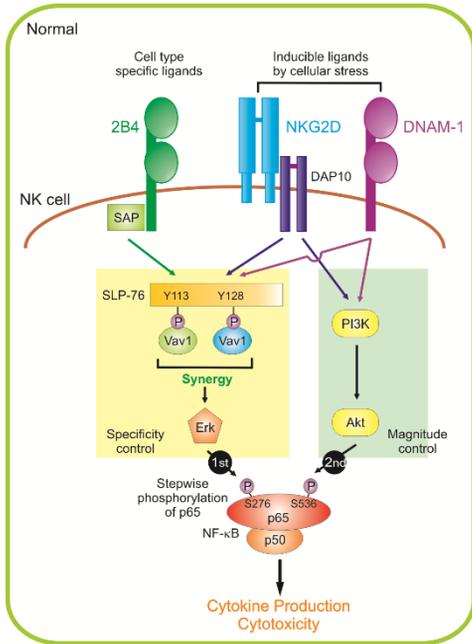


Fig. 78. Proposed mechanism of NF- κ B activation via coactivation receptors on NK cells.

NF- κ B activation in NK cells required the coordinated engagement of coactivation receptors, such as 2B4 and NKG2D or 2B4 and DNAM-1. This combination was required to provide complementary and independent signals leading to Vav1-dependent synergistic signaling involving PLC- γ 2 and Erk. Further, signals from synergizing receptors converged on NF- κ B p65 subunit through selective phosphorylation of p65 serine residues, particularly at serine 276 via Vav1-Erk and at serine 536 via PI3K-Akt pathway, which was crucial to optimal activation of NF- κ B. The requisite PI3K-Akt signal was primarily mediated by the engagement of NKG2D or DNAM-1, which recognizes ligands induced by cellular stress. Vav1 controlled downstream p65 phosphorylation and NF- κ B activation, suggesting that distinct signaling checkpoints at the level of Vav1 and p65 regulate NF- κ B activation. In support, Vav1-dependent synergistic signaling was required for the phosphorylation of p65 at serine 536 by Akt pathway, which was evident in SAP-deficient XLP1 NK cells following coactivation, which exhibited impaired p65 phosphorylation, nuclear translocation, NF- κ B activation and downstream NF- κ B-dependent functions.

the inhibition of Vav1 by inhibitory receptor or Vav1 knockdown resulted in abrogation of NF- κ B activation during coactivation.

In addition, our study revealed that Vav1-dependent synergistic signaling was crucial but insufficient for full NF- κ B activation. The outcome of NF- κ B activation also relied on signal integration by NF- κ B p65 subunit via specific phosphorylation at regulatory serine residues. Post-translational modification of NF- κ B subunit has long been appreciated as an important regulatory mechanism, dictating its transcriptional activity and target gene specificity (25). Among others, phosphorylation of the p65 subunit plays a key role in determining the specificity, strength and duration of NF- κ B-dependent gene programs (24, 27). Thus, such modification likely serves to fine-tune NF- κ B transcriptional activity, rather than functions as a simple on-off switch (27). Although the possibility of signal integration by other proteins cannot be excluded, our results reveal that synergistic NF- κ B activation is kept in check at the level of p65 by the requirement for a PI3K-Akt signal, in addition to the Vav1-Erk signal. In a T cell study, Akt was shown to fine-tune NF- κ B signaling and transcription during CD3 and CD28 stimulation, in part through its effects on p65 (35). Of interest, we observed that the requisite PI3K-Akt signal for NF- κ B activation was principally mediated by stimulation through NKG2D or DNAM-1, but not 2B4. The natural ligands for NKG2D (MICA/B and the family of ULBPs) and DNAM-1 (CD155 and CD112) are frequently upregulated on cells under stress conditions associated with malignant transformation or viral infection (36, 37). Thus, it is likely that NK cell stimulation by NKG2D or DNAM-1 in combination with 2B4 could trigger tailored NF- κ B responses according to the expression levels of cognate ligands on stressed cells.

The regulation of NF- κ B activation at multiple levels may serve as a safeguard to prevent inadvertent gene transcription. The identification of distinct signaling checkpoints “upstream” at SLP-76-Vav1 and “downstream” at p65, as shown here, is consistent with the tight control of NF- κ B activation. Similar patterns of SLP-76, Vav1, and p65 phosphorylation were induced by synergy among NKG2D, 2B4, and DNAM-1, suggesting a common logic for signal coordination among coactivation receptors. Moreover, the dependence of synergistic p65 phosphorylation and NF- κ B activation on “upstream” Vav1, and the defects of such regulation in XLP1 NK cells, suggests that stepwise signaling checkpoints at the level of Vav1 and p65 control NF- κ B activation. Supporting this, p65 phosphorylation at serine 536 by PI3K-Akt pathway was apparent upon stimulation with NKG2D and 2B4, but not NKG2D alone although Akt phosphorylation by NKG2D crosslinking was not enhanced by co-crosslinking with 2B4. Likewise, in XLP1 NK cells, Vav1-dependent synergistic signaling was prerequisite to mediate pS536-p65 by Akt pathway.

Our present analysis of XLP1 NK cells provides an insight into the mechanism by which SAP deficiency affects NF- κ B activation and NK cell functions during coactivation. Mutations in the *SH2D1A* gene, which result in the lack or dysfunction of SAP, form the genetic basis of XLP1 (29-31). XLP1 patients are particularly susceptible to EBV infection. Among the defects in XLP1 lymphocytes, the inability of XLP1 NK and CD8⁺ T cells to eliminate EBV-infected B cells largely accounts for the persistence of infected B cells, fulminant mononucleosis, and B-cell lymphoma (38, 39). SAP is an adaptor protein required for transmitting activation signals elicited through SLAM family receptors, including 2B4 (40). Accordingly,

SAP-deficient XLP1 NK cells fail to be activated through 2B4 and show defects in 2B4-mediated killing of EBV-infected B cells and production of IFN- γ (32, 33). Here we found that SAP deficiency impedes synergistic NF- κ B activation by NKG2D and 2B4 coactivation at the level of Vav1 and Vav1-dependent downstream signals, such as Erk. This Erk pathway activation was required for cytotoxic degranulation and crucial to phosphorylate p65 at key serine residue (S276-p65) for NF- κ B activation. These results are consistent with the dependence of 2B4-mediated activation on SAP through Fyn-induced phosphorylation of Vav1 (8). However, possible contribution of SAP deficiency to other transcription factors including IRFs cannot be excluded. In SAP deficiency, 2B4 was shown to recruit protein tyrosine phosphatases (e.g., SHIP-1) and impair the activity of co-engaged activating receptors by delivering inhibitory signals (8, 30, 32). A recent study showed that this inhibitory function of 2B4 in XLP1 NK cells is confined to ITAM-dependent signaling pathways and does not affect the activity of non-ITAM-associated NKG2D and DNAM-1 (41). In support, upon NKG2D and 2B4 coengagement, NKG2D-dependent phosphorylation of Vav1 and Akt were preserved, whereas synergistic signals through combined Vav1 phosphorylation were abrogated in XLP1 NK cells (Fig. 66-73). In SAP-null murine NK cells, 2B4 could repress NKG2D (42) which, unlike human NKG2D, recruits both DAP10 and ITAM-associated DAP12 (23). Thus, we speculate that 2B4-mediated inhibition is selective to the activating receptors coengaged although the exact mechanism underlying this selectivity in inhibition remains to be determined.

It has been shown that NK cells become 'primed' upon exposure to cytokines such as IL-2 or IL-15 and, in turn, have enhanced reactivity against target cells, a situation that likely occurs during the course of infection and transformation.

Such cytokine-stimulated NK cells respond to the engagement of single activating receptor (e.g., NKG2D, 2B4) for effector functions (5, 6, 28, 43), probably due to a lower threshold for activation than resting NK cells. Supporting this, the proportion of cells expressing IFN- γ was significantly increased in IL-2 stimulated NK cells following stimulation with NKG2D or 2B4 alone, correlating with enhanced NF- κ B activation (Fig. 79A,B). Further, IFN- γ expression and NF- κ B activation by NKG2D and 2B4 coengagement were also enhanced after IL-2 stimulation, suggesting that synergistic coactivation exists, even in the context of a high IL-2 environment. We found a gradual decrease in c-Cbl but not Vav1 and a marginal phosphorylation of Vav1 after IL-2 stimulation (Fig. 80A, B), suggesting downregulation of c-Cbl as a potential mechanism that relieves the requirement for coactivation. However, the involvement of other regulatory mechanism(s) cannot be excluded.

In conclusion, we provide evidence that, unlike ITAM-dependent pathways, such as those triggered by antigen-specific receptors of adaptive immune cells and Fc γ receptor CD16 in NK cells, a single coactivation receptor such as 2B4 or NKG2D is incompetent to induce NF- κ B activation. Instead, it requires complementation of coactivation receptors with distinct signaling properties to achieve proper specificity and optimal magnitude of NF- κ B activation. Because PI3K-Akt and Erk pathways are often induced by diverse NF- κ B-activating stimuli (26, 27) and involved in site-selective phosphorylation of p65, the model for coordinated NF- κ B activation through combined p65 phosphorylation described here may apply to other NF- κ B-activating stimuli in various cell types.

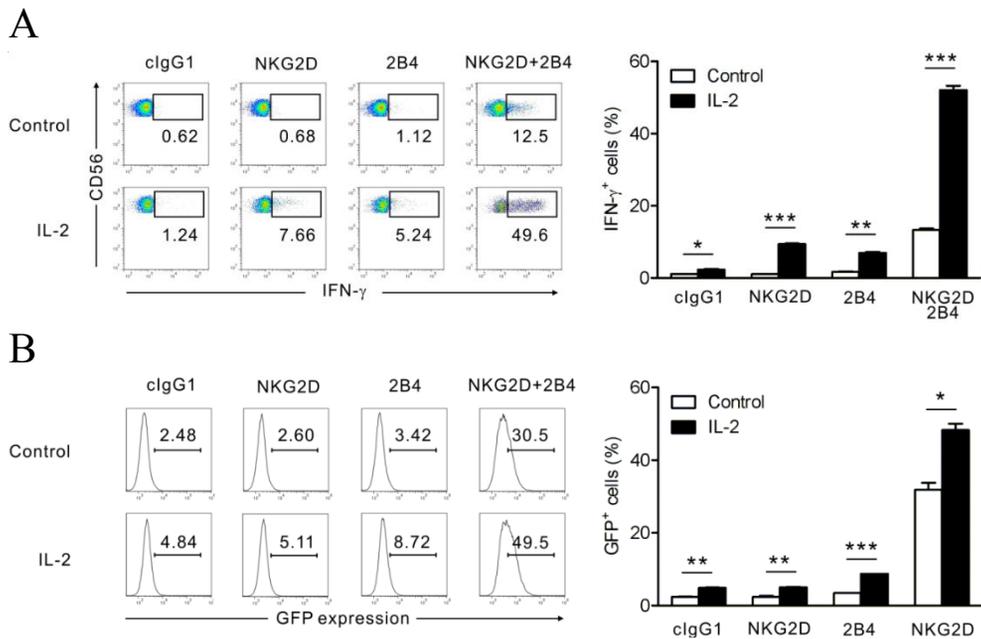


Fig. 79. Preactivation with IL-2 enhances IFN- γ expression and NF- κ B activation.

(A) Frequency of NK cells that displayed IFN- γ expression after pretreatment of PBMCs with or without IL-2 (400 U/ml) and then stimulation with P815 target cells as indicated. After incubation for 6 h, cells were stained and analyzed by flow cytometry. Representative result (left) and statistical bar charts (right) are shown. Values represent mean \pm s.d.

(B) NKL- κ B-GFP cells were rested or pretreated with IL-2 (400 U/ml) for 20 h and then stimulated with plate-immobilized mAbs specific for NKG2D and/or 2B4 for 6 h in the absence or presence of IL-2 (400 U/ml). GFP expression in NKL- κ B-GFP cells was analyzed by flow cytometry, and representative result (left) and statistical bar charts (right) are shown. Values represent mean \pm s.d. * P <0.05; ** P <0.01; *** P <0.001 (two-sided Student's t -test). Data are representative of three independent experiments.

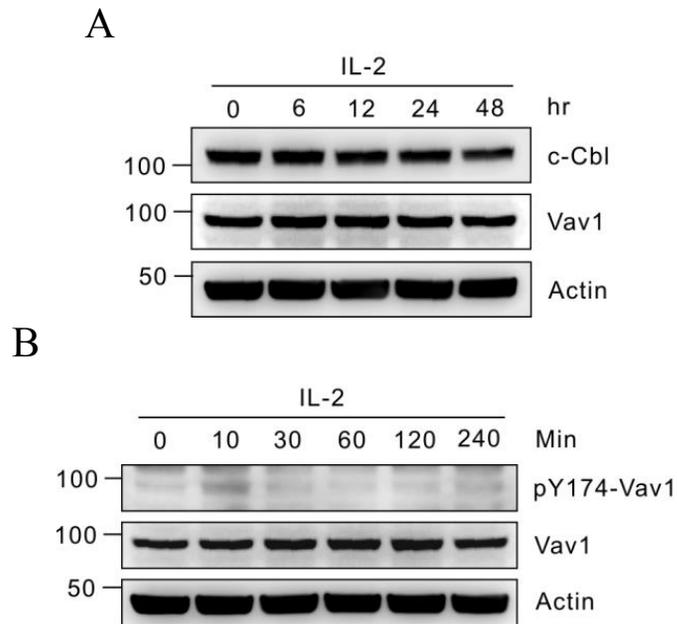


Fig. 80. Preactivation with IL-2 enhances the responsiveness of NK cells for IFN- γ expression and NF- κ B activation.

(A and B) Rested NKL cells were stimulated with IL-2 (400 U/ml) for the indicated time. Cell lysates were immunoblotted for c-Cbl, Vav1, or actin (A) or phospho-Vav1 at tyrosine 174 (pY174), Vav1, or actin (B). Data are representative of three independent experiments.

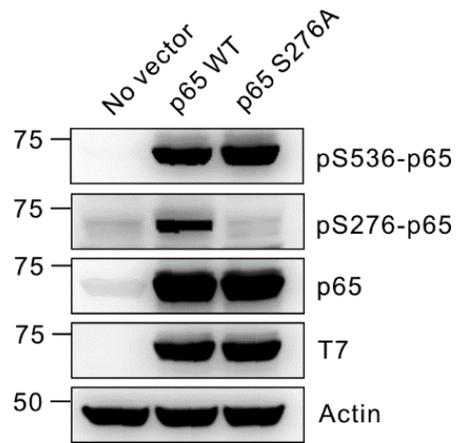


Fig. 81. The specificity of the antibody against p65 at serine 276.

293T cells were transfected with pEV3s-T7-RelA or pEV3s-T7-RelA S276A expression vector. After 48 h incubation, cell lysates were immunoblotted for phospho-p65 at serine 536 (pS536), phospho-p65 at serine 276 (pS276), p65, T7, or actin. Data are representative of three independent experiments.

Table 1. List of primers used for real-time PCR analyses of the indicated genes.

Functional category	Protein (<i>Gene</i>)	Primer (5'-3')
Cytokine	IFN- γ (<i>IFNG</i>)	Forward: GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC Reverse: CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG
	TNF- α (<i>TNF</i>)	CAGAGGGCCTGTACCTCATC GGTTGAGGGTGTCTGAAGGA
Chemokine	MIP-1 α (<i>CCL3</i>)	GCTGACTACTTTGAGACGAGC CCAGTCCATAGAAGAGGTAGC
	MIP-1 β (<i>CCL4</i>)	CCAAACCAAAAAGAAGCAAGC AGAAAACAGTGACAGTGGACC
	RANTES (<i>CCL5</i>)	ATCCTCATTTGCTACTGCCCTC GCCACTGGTGTAGAAATACTCC
Cytolytic pathway	Granzyme B (<i>GZMB</i>)	CCCTGGGAAAACACTCACACA CACAACCTCAATGGTACTGTCTGT
	Perforin (<i>PRF1</i>)	CGCCTACCTCAGGCTTATCTC CCTCGACAGTCAGGCAGTC
Death receptor apoptotic pathway	FASL (<i>FASLG</i>)	CTTGGTAGGATTGGGCCTGG TGTGTGCATCTGGCTGGTAG
	TRAIL (<i>TNFSF10</i>)	CCGGCTGCCTGGCTGACTTAC TCAGCACGCAGGTCTGTCCC
NF- κ B pathway	I κ B α (<i>NFKBIA</i>)	TGGCCTTCCTCAACTTCCAGAACA CTCAGCAATTCTGGCTGGTTGGT
	p65 (<i>RELA</i>)	GAAGAAGAGTCCTTTCAGCG GGGAGGACGTAAGGGATAG
	BCL10 (<i>BCL10</i>)	CCCCTCCGCCTCCTCTCCTT GGCGTTCTTCCGGGTCCG
Apoptosis	BCL-XL (<i>BCL2L1</i>)	GACTGAATCGGAGATGGAGACC GCAGTTCAAACTCGTCGCCT
	MCL1 (<i>MCL1</i>)	CATTCTGATGCCACCTTCT TCGTAAGGACAAAACGGGAC
IL-2 receptor	CD122 (<i>IL2RB</i>)	TCATCATCTTAGTGTACTTGCTGATCA GGTGTTACACTTCAGGACCTTCTTC
	CD132 (<i>IL2RG</i>)	GGAGCAATACTTCAAAAAGAGAATCCT CCCATGGAGCCAACAGAGAT
Cytolytic granule exocytosis	Munc13-4 (<i>UNC13D</i>)	GCCAGGCCATCAAGATAAAGG CTCGGGGGAGAAGTGGTG
	Rab27a (<i>RAB27A</i>)	AGCAGGGCAGGAGAGGTTTCGTA TGCTATGGCTTCCTCCTCTTTCAC
	Syntaxin11 (<i>STX11</i>)	ACAGGTTTCCTTCTCCATCG TGCTGGTCATATTGCTTGGA
House keeping gene	β -actin (<i>ACTB</i>)	ACTCCATCATGAAGTGTGACG CATACTCTGCTTGCTGATCC

III-6. References

1. Long, E.O., Kim, H.S., Liu, D., Peterson, M.E. & Rajagopalan, S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu. Rev. Immunol.* **31**, 227-258 (2013).
2. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat. Immunol.* **9**, 503-510 (2008).
3. Moretta, L. *et al.* Surface NK receptors and their ligands on tumor cells. *Semin. Immunol.* **18**, 151-158 (2006).
4. Kim, H.S., Das, A., Gross, C.C., Bryceson, Y.T. & Long, E.O. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity* **32**, 175-186 (2010).
5. Cooper, M.A. *et al.* Cytokine-induced memory-like natural killer cells. *Proc. Natl. Acad. Sci. USA* **106**, 1915-1919 (2009).
6. Romee, R. *et al.* Cytokine activation induces human memory-like NK cells. *Blood* **120**, 4751-4760 (2012).
7. Bloch-Queyrat, C. *et al.* Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn. *J. Exp. Med.* **202**, 181-192 (2005).
8. Dong, Z. *et al.* The adaptor SAP controls NK cell activation by regulating the enzymes Vav-1 and SHIP-1 and by enhancing conjugates with target cells. *Immunity* **36**, 974-985 (2012).

9. Lanier, L.L. Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* **9**, 495-502 (2008).
10. Ma, C.S., Nichols, K.E. & Tangye, S.G. Regulation of cellular and humoral immune responses by the SLAM and SAP families of molecules. *Annu. Rev. Immunol.* **25**, 337-379 (2007).
11. Upshaw, J.L. *et al.* NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat. Immunol.* **7**, 524-532 (2006).
12. Shibuya, A., Lanier, L.L. & Phillips, J.H. Protein kinase C is involved in the regulation of both signaling and adhesion mediated by DNAX accessory molecule-1 receptor. *J. Immunol.* **161**, 1671-1676 (1998).
13. Nabekura, T. *et al.* Costimulatory molecule DNAM-1 is essential for optimal differentiation of memory natural killer cells during mouse cytomegalovirus infection. *Immunity* **40**, 225-234 (2014).
14. Fauriat, C., Long, E.O., Ljunggren, H.G. & Bryceson, Y.T. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* **115**, 2167-2176 (2010).
15. Fehniger, T.A. *et al.* Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* **162**, 4511-4520 (1999).
16. Schulze-Luehrmann, J. & Ghosh, S. Antigen-receptor signaling to nuclear factor

- kappa B. *Immunity* **25**, 701-715 (2006).
17. Vallabhapurapu, S. & Karin, M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu. Rev. Immunol.* **27**, 693-733 (2009).
 18. Orange, J.S. *et al.* Deficient natural killer cell cytotoxicity in patients with IKK-gamma/NEMO mutations. *J. Clin. Invest.* **109**, 1501-1509 (2002).
 19. Pannicke, U. *et al.* Deficiency of innate and acquired immunity caused by an IKBKB mutation. *N. Engl. J. Med.* **369**, 2504-2514 (2013).
 20. Gross, O. *et al.* Multiple ITAM-coupled NK-cell receptors engage the Bcl10/Malt1 complex via Carma1 for NF-kappaB and MAPK activation to selectively control cytokine production. *Blood* **112**, 2421-2428 (2008).
 21. Pandey, R., DeStephan, C.M., Madge, L.A., May, M.J. & Orange, J.S. NKp30 ligation induces rapid activation of the canonical NF-kappaB pathway in NK cells. *J. Immunol.* **179**, 7385-7396 (2007).
 22. Diefenbach, A. *et al.* Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat. Immunol.* **3**, 1142-1149 (2002).
 23. Gilfillan, S., Ho, E.L., Cella, M., Yokoyama, W.M. & Colonna, M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat. Immunol.* **3**, 1150-1155 (2002).
 24. Hayden, M.S. & Ghosh, S. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev.* **26**, 203-234 (2012).
 25. Perkins, N.D. Integrating cell-signalling pathways with NF-kappaB and IKK

- function. *Nat. Rev. Mol. Cell. Biol.* **8**, 49-62 (2007).
26. Viatour, P., Merville, M.P., Bours, V. & Chariot, A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem. Sci.* **30**, 43-52 (2005).
 27. Oeckinghaus, A., Hayden, M.S. & Ghosh, S. Crosstalk in NF-kappaB signaling pathways. *Nat. Immunol.* **12**, 695-708 (2011).
 28. Bryceson, Y.T., March, M.E., Ljunggren, H.G. & Long, E.O. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107**, 159-166 (2006).
 29. Coffey, A.J. *et al.* Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat. Genet.* **20**, 129-135 (1998).
 30. Sayos, J. *et al.* The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* **395**, 462-469 (1998).
 31. Nichols, K.E. *et al.* Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc. Natl. Acad. Sci. USA.* **95**, 13765-13770 (1998).
 32. Parolini, S. *et al.* X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. *J. Exp. Med.* **192**, 337-346 (2000).
 33. Tangye, S.G., Phillips, J.H., Lanier, L.L. & Nichols, K.E. Functional

- requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. *J. Immunol.* **165**, 2932-2936 (2000).
34. Kim, H.S. & Long, E.O. Complementary phosphorylation sites in the adaptor protein SLP-76 promote synergistic activation of natural killer cells. *Sci. Signal.* **5**, ra49 (2012).
35. Cheng, J., Phong, B., Wilson, D.C., Hirsch, R. & Kane, L.P. Akt fine-tunes NF-kappaB-dependent gene expression during T cell activation. *J. Biol. Chem.* **286**, 36076-36085 (2011).
36. Raulet, D.H., Gasser, S., Gowen, B.G., Deng, W. & Jung, H. Regulation of ligands for the NKG2D activating receptor. *Annu. Rev. Immunol.* **31**, 413-441 (2013).
37. Cerboni, C. *et al.* The DNA Damage Response: A Common Pathway in the Regulation of NKG2D and DNAM-1 Ligand Expression in Normal, Infected, and Cancer Cells. *Front. Immunol.* **4**, 508 (2014).
38. Hislop, A.D. *et al.* Impaired Epstein-Barr virus-specific CD8+ T-cell function in X-linked lymphoproliferative disease is restricted to SLAM family-positive B-cell targets. *Blood* **116**, 3249-3257 (2010).
39. Palendira, U. *et al.* Molecular pathogenesis of EBV susceptibility in XLP as revealed by analysis of female carriers with heterozygous expression of SAP. *PLoS Biol.* **9**, e1001187 (2011).
40. Veillette, A. Immune regulation by SLAM family receptors and SAP-related adaptors. *Nat. Rev. Immunol.* **6**, 56-66 (2006).

41. Meazza, R. *et al.* XLP1 inhibitory effect by 2B4 does not affect DNAM-1 and NKG2D activating pathways in NK cells. *Eur. J. Immunol.* **44**, 1526-1534 (2014).
42. Dong, Z. *et al.* Essential function for SAP family adaptors in the surveillance of hematopoietic cells by natural killer cells. *Nat. Immunol.* **10**, 973-980 (2009).
43. Billadeau, D.D., Upshaw, J.L., Schoon, R.A., Dick, C.J. & Leibson, P.J. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat. Immunol.* **4**, 557-564 (2003).
44. Dupuy, S. *et al.* Human Herpesvirus 8 (HHV8) sequentially shapes the NK cell repertoire during the course of asymptomatic infection and Kaposi sarcoma. *PLoS Pathog.* **8**, e1002486 (2012).
45. Brandt, C.S. *et al.* The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J. Exp. Med.* **206**, 1495-1503 (2009).
46. Arnon, T.I. *et al.* Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur. J. Immunol.* **31**, 2680-2689 (2001).
47. Martinez, E. *et al.* NKG2D-dependent cytotoxicity is controlled by ligand distribution in the target cell membrane. *J. Immunol.* **186**, 5538-5542 (2011).
48. Booth, C. *et al.* X-linked lymphoproliferative disease due to SAP/SH2D1A deficiency: a multicenter study on the manifestations, management and outcome of the disease. *Blood* **117**, 53-62 (2011).
49. Chaigne-Delalande, B. *et al.* Mg²⁺ regulates cytotoxic functions of NK and CD8 T cells in chronic EBV infection through NKG2D. *Science* **341**, 186-191 (2013).

50. Imai, C., Iwamoto, S. & Campana, D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* **106**, 376-383 (2005).
51. Fujisaki, H. *et al.* Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res.* **69**, 4010-4017 (2009).
52. Spooren, A. *et al.* Hunting for serine 276-phosphorylated p65. *J. Biomed. Biotechnol.* **2010**, 275892 (2010).

국문 초록

자연살해세포는 바이러스 감염과 종양 형성을 제어함에 있어 중요한 역할을 하는 세포용해활성을 가짐으로 선천 면역 림프구 중에서도 구별된다. 자연살해세포는 세포독성 과립을 분비하여 바이러스에 감염되었거나 형질 전환된 세포를 죽일 뿐 아니라, 시토카인 (예, IFN- γ , TNF- α) 과 케모카인 (예, MIP-1 α , MIP-1 β) 을 분비하여 다양한 면역반응도 조절한다. 따라서 자연살해세포의 기능장애는 다양한 질병과 연관되어 있고, 자연살해세포의 기능 조절을 적용한 치료법이 개발될 가능성도 있다. 그러나 자연살해세포 활성 조절 기전에 대한 정보의 부족이 치료법의 개발 및 발전을 어렵게 하고 있다. 그러므로 자연살해세포의 기능을 조절하는 정확한 분자 기전에 대한 이해는 성공적인 자연살해세포 기반 치료법 개발에 도움을 줄 수 있다.

자연살해세포의 작동 기능은 활성화수용체 또는 억제수용체와 표적세포 리간드 사이의 결합으로 유발된다. 활성화수용체와 억제수용체의 종합적인 신호가 자연살해세포의 활성을 결정하는 것으로 알려져 있다. 그러나 억제신호가 없는 경우에도 단독 활성화수용체에 의한 활성 신호는 휴지기 자연살해세포를 충분히 활성화 시키지 못한다. 자연살해세포의 기능을 위해 단독 활성화수용체가 아닌 복합 활성화수용체로부터의 신호전달이 필요하다. 그리고 그 중에 특정 조합만이 자연살해세포 활성의 부가적 또는 시너지 증가를 유도할 수 있다. 이러한 이유로 자연살해세포의 시너지 활

성을 조절하는 신호전달 기전의 연구는 임상 목적의 자연살해세포 활용에 결정적이다. 본 연구는 면역세포기능에 있어 두 가지 중요한 신호전달 물질인 GSK-3 β 와 NF- κ B의 자연살해세포의 시너지 활성 조절을 살펴 보았고, 이들의 다른 신호전달 경로와 상호작용 기전을 알아보았다.

대표적인 ITAM-비결합 활성 수용체인 NKG2D와 2B4 활성수용체를 조합한 자연살해세포 시너지 활성 모델에서 GSK-3 β 는 시너지 활성에 의한 세포독성과 시토카인 분비 활성을 억제하고 있었다. NKG2D와 2B4를 단독 또는 조합으로 자극했을 때 GSK-3 β 의 억제 인산화가 유도되었고, 인산화의 정도는 자연살해세포의 활성 수준과 일치하였다. siRNA 또는 약리적 방법을 이용한 GSK-3 β 의 억제는 자연살해세포의 기능을 증가시켰지만 GSK-3 α 의 억제는 영향이 없었다. 이러한 GSK-3 β 의 억제 기능은 자체의 인산화 활성화에 의존적이었다. GSK-3 β 의 자연살해세포 조절 기능은 NKL 자연살해세포주 및 일차 자연살해세포의 공통 기전이었다. 그리고 ITAM-결합 및 비결합 활성수용체에 의한 자연살해세포의 활성이 GSK-3 β 에 의해 조절되었다.

NF- κ B는 다양한 면역 반응에 중요한 잘 알려진 전사 인자이다. 그럼에도 표적세포를 인지할 때 복합적인 활성수용체에 의해 활성화되는 자연살해세포에서의 역할은 알려지지 않았다. NKG2D, 2B4, DNAM-1 등의 시너지 조합은 자연살해세포의 시너지 활성으로 연결되기에 충분한 NF- κ B 신호전달 경로의 시너지 활성을 유도하였지만, 단독 수용체 자극은 충분하지 않았다. 수용체 조합에 의한 NF- κ B의 활성화는 1차 확인점인

Vav1 인산화 단계에서 조절되었고, 이 Vav1 의존적인 시너지 신호 전달은 또 다른 PI3K-Akt 신호 전달과 협력하여 2차 확인점인 p65의 시너지 인산화를 단계적으로 유도하였다. SAP adaptor의 변이로 인해 2B4 수용체 신호 전달이 결여된 만성 림프증식성 질환 자연살해세포로 확인한 바, NF- κ B 활성을 위한 단계적인 확인점을 통한 신호전달 기전을 제시하였다.

새롭게 발견된 복합적 수용체 자극 시의 신호 전달 기전은 자연살해세포 활성의 특이성과 강도를 결정하는 수용체 간 협력에 대한 새로운 시각 및 자연살해세포 기반 치료 전략 수립에 대한 근거를 제시한다.

핵심어: 자연살해세포, 시너지 활성, 수용체 신호 전달, GSK-3 β , NF- κ B, 만성 림프증식성 질환

학번: 2008-30923